THE ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN PHYSIOLOGY AND PATHOLOGY OF REPRODUCTIVE FUNCTION

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DEDICATION

"Aum Shree Satya Sai Gaṅeshaya Namah".

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ABSTRACT OF THE THESIS:

Vascular endothelial growth factor (VEGF) is a multifunctional cytokine. It has potent angiogenic properties and makes endothelial cells hyperpermeable to plasma proteins.

In the clinical studies that constitute this thesis, serum concentrations of VEGF were measured by enzyme immunoassay. Initial studies documented fluctuations of serum VEGF in women during the normal menstrual cycle and their relationship to pelvic blood flow, as assessed by colour Doppler ultrasonography.

The hypothesis that VEGF plays a role in the pathogenesis of ovarian hyperstimulation syndrome (OHSS) was explored. Women who recruit an excessive number of follicles overexpress VEGF, which may result in a fluid shift into the extravascular space, which characterises the syndrome of OHSS. Women with polycystic ovaries (PCO), who are at high risk of OHSS, and women who developed OHSS had higher serum concentrations of VEGF and pelvic blood flow velocities than women with normal ovaries and than those who did not develop OHSS. Pregnancy was also associated with higher circulating VEGF concentrations than non-pregnant states.

A further study explored the contribution of the reproductive tract to circulating VEGF concentrations. Women who had their uterus and ovaries in situ had higher VEGF concentrations than women who had previously undergone hysterectomy and oophorectomy. Treatment of postmenopausal women with hormone replacement therapy (HRT) was associated with higher serum VEGF than no treatment with HRT.

The hypothesis that raised serum VEGF concentrations in women with PCO results from excess release by granulosa cells was tested in in-vitro studies. VEGF released by cultured human luteinised granulosa cells was measured by a sensitive "sandwich" enzyme immunoassay. Release of VEGF into the culture medium was augmented by incubation of cells with hCG, FSH and

LH. Co-incubation with insulin augmented hCG - stimulated release of VEGF. More VEGF was released from granulosa cells obtained from women with PCO than from granulosa cells obtained from women with normal ovaries. These results were consistent with the hypothesis that raised serum VEGF concentrations in women with PCO results from excessive VEGF release by granulosa cells.

The reproductive tract contributes significantly to circulating VEGF concentrations. The presence of PCO, OHSS, pregnancy and treatment with HRT were all associated with elevated serum VEGF concentrations.

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PUBLICATIONS ORIGINATING FROM THE WORK CONTAINED IN THE THESIS:

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ABBREVIATIONS:

BMI	body mass index
CL	corpus luteum
E_2	oestradiol
EDRF	endothelium derived relaxing factor
ET	embryo transfer
FISH	fluorescent in - situ hybridisation
Flt-1	fms like tyrosine kinase receptor
FSH	follicle stimulating hormone
GnRH	gonadotrophin releasing hormone
hCG	human chorionic gonadotrophin
HGF	hepatocyte growth factor
Ig	immunoglobulins
IGF	insulin like growth factor
IGFBP	insulin like growth factor binding protein
IL	interleukins
IVF	in - vitro fertilisation
KDR/Flk - 1	kinase domain region/fetal liver kinase receptor
NO	nitric oxide
OHSS	ovarian hyperstimulation syndrome
PCO	polycystic ovaries
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
PDECGF	platelet derived endothelial cell growth factor
PDGF	platelet derived growth factor
PGE_2	prostaglandin E_2
PlGF	placental growth factor
TGF	transforming growth factor
TNF	tumour necrosis growth factor
VEGF	vascular endothelial growth factor
VPF	vascular permeability factor
VSM	vascular smooth muscle cells

Introduction

INTRODUCTION:

Section 1: Biology of vascular endothelial growth factor

Angiogenesis is the formation of new blood vessels or extension of pre-existing ones. It involves endothelial cell division and migration, selective degradation of vascular basement membranes and surrounding extracellular matrix (Folkman and Shing, 1992). It is essential for organ development and differentiation in the embryo (Hamilton et al., 1962, Gilbert, 1988). In the adult, however, turnover rates of vascular endothelial cells are extremely low. Angiogenesis is therefore generally a slow process except during wound healing and in the female reproductive system (Folkman and Klagsbrun, 1987), where the need for additional vasculature is constantly imposed by the cyclical evolution of transient structures and repair of damaged tissue (Folkman, 1991). Angiogenesis is also fundamental to the pathogenesis of a variety of disorders such as tumours, proliferative retinopathies, human rheumatoid arthritis, psoriasis and age related macular degeneration (Folkman et al., 1987, 1991).

Angiogenesis is thought to be initiated by local activation of genes encoding diffusible angiogenic factors or by release of preformed growth factors from their stores. The secreted growth factors initiate a cascade of responses, which result in the growth of new capillaries towards the site of the angiogenic signal. Several growth factors are implicated as potential regulators of angiogenesis such as vascular endothelial growth factor (VEGF), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF) (Esch et al., 1985), epidermal growth factor (EGF; Nelson et al., 1991)), transforming growth factor-alpha (TGF-alpha; Schreiber et al., 1986), TGF-beta (Roberts et al., 1986), hepatocyte growth factor (HGF), prostaglandin E_2 (PGE_2), monobutyrin (Klagsbrun and D'Amore, 1991, Folkman and Shing, 1992), angiogenin (Fett et al., 1985), PD-ECGF, tumour necrosis factor-alpha (TNF-alpha; Leibovich et al., 1987), interleukin - alpha (IL-8), platelet derived endothelial cell growth factor (PD -ECGF), placental derived growth factor (PlGF) etc. Some of these factors directly stimulate endothelial cell growth, others require paracrine release of direct-acting

growth factors. Although these growth factors are able to promote angiogenesis, their exact role in physiological and pathological regulation of blood vessel growth is not known (Klagsbrun et al., 1991, Folkman, 1992, Ferrara and Keyt, 1997).

In this thesis the role of VEGF in angiogenesis in the reproductive tract and its association with reproductive function is explored. Since Doppler ultrasonography is helpful for determining blood flow velocities within newly formed or pre-existing blood vessels, it was used as a supplementary guide to detect angiogenesis within the pelvic blood vessels thought to be related to changes in circulating VEGF.

1.1 Biological activities of VEGF:

Several recent publications have suggested a pivotal role for VEGF in the regulation of normal and abnormal angiogenesis (Ferrara, 1993). VEGF is an endothelial cell mitogen and a multifunctional cytokine with potent angiogenic properties. It acts on micro and macrovascular endothelial cells of arteries, veins and lymphatics but it is devoid of appreciable mitogenic activity on other cell types (Ferrara et al., 1989, Leung et al., 1989, Connolly et al., 1989, Keck et al., 1989, Plouet et al., 1989, Conn et al., 1990, Pepper et al., 1994). VEGF was originally discovered as a tumour secreted protein that rendered microvessels, primarily postcapillary venules and small veins, hyperpermeable to circulating macromolecules (Senger et al., 1993, Dvorak et al., 1995). Subsequently it was learnt that VEGF acted directly on cultured endothelial cells (Fig 9.1.1), to induce transient accumulation of cytoplasmic calcium (Brock et al., 1991), change of cell shape (Senger et al., 1993), cell division (Connolly et al., 1989, Ferrara et al., 1989, Gospodarowicz et al., 1989, Plouet et al., 1989, Tischer et al., 1989, Ferrara et al., 1991, Houck et al., 1991, Sioussat et al., 1993) and migration (Favard et al., 1991) (Fig 9.1.2). VEGF altered the pattern of endothelial cell gene expression and induced angiogenesis in vivo. VEGF promotes angiogenesis by inducing confluent vascular endothelial cells to invade collagen gels and forming capillary - like structures (Pepper et al., 1992).

The crucial role of VEGF in differentiation and development of the vascular system is evidenced by the recent demonstration in a transgenic mouse, of the effect of loss of a single VEGF allele which resulted in embryonic lethality (Carmeliet et al., 1996, Ferrara et al., 1996). VEGF induced therapeutic angiogenesis within the coronary (Banai et al., 1994, Pearlman et al., 1995, Harada et al., 1996) and limb (Takeshita et al., 1994 (a, b), Takeshita et al., 1996) arteries of animals provides further evidence of its important role in angiogenesis.

Besides stimulating proliferation and migration of endothelial cells, VEGF makes these cells hyperpermeable so that plasma proteins leak into the extravascular space. VEGF is therefore also known as *vascular permeability*

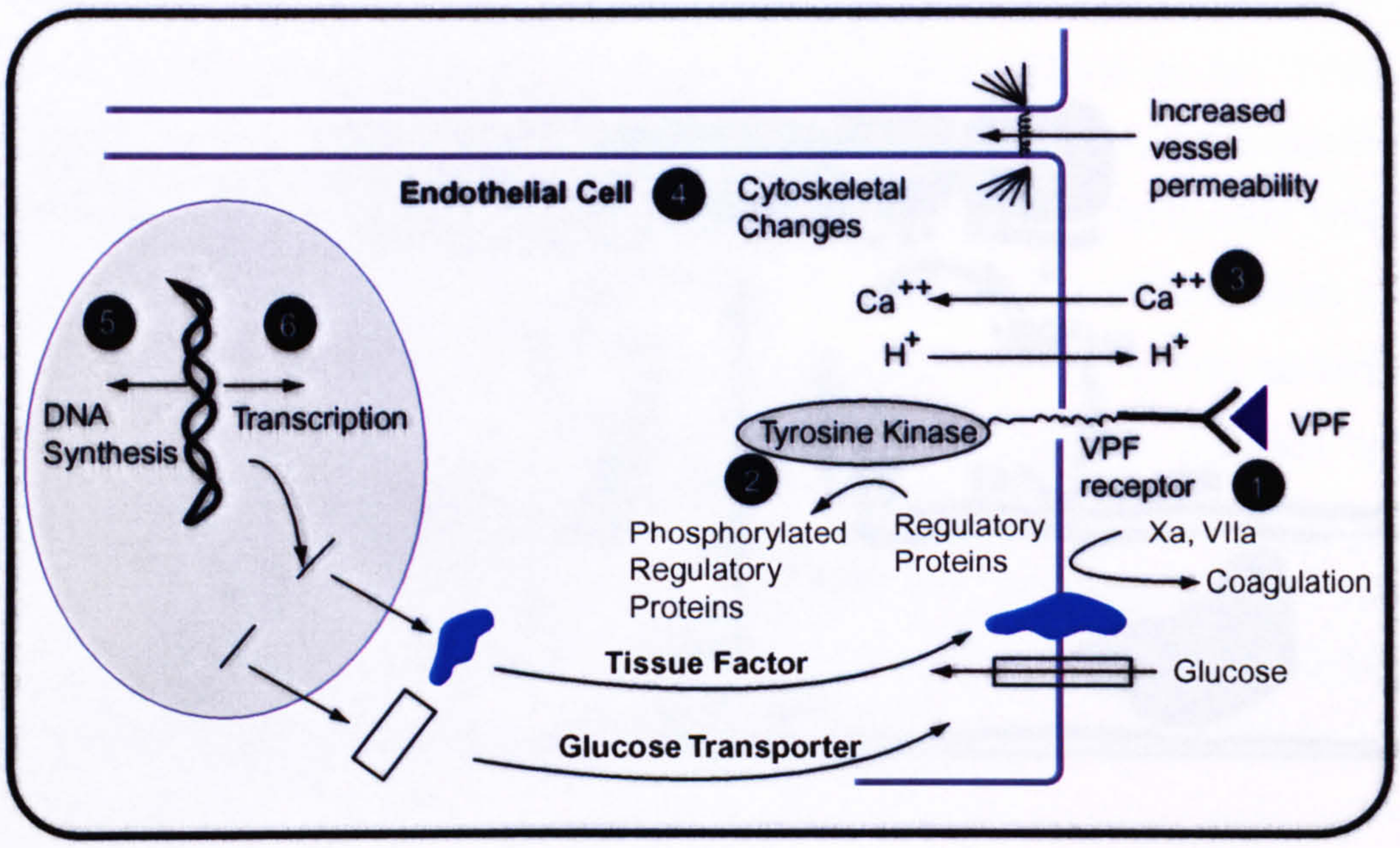


Figure 9.1.1

The mode of action and interaction of VEGF/VPF on endothelial cells.

1: VPF binds to high-affinity cell surface receptors.

2: Tyrosine kinase activity, presumably associated with the receptor itself, is activated upon VPF binding. Regulatory proteins are phosphorylated on tyrosine residues.

3: Intracellular $Ca^{2}+$, pH and inositol trisphosphate increase.

4: Cytoskeletal changes causing cellular contraction leads to increased vascular permeability.

5: DNA synthesis and mitosis are initiated by phosphotyrosine residues which enhance receptor catalytic activity or provide docking sites for dowstream signalling protein.

6: The genes for tissue factor and glucose transporter are activated. Glucose transport is increased. Tissue factor can interact with coagulation factors to initiate coagulation (Connolly 1991).

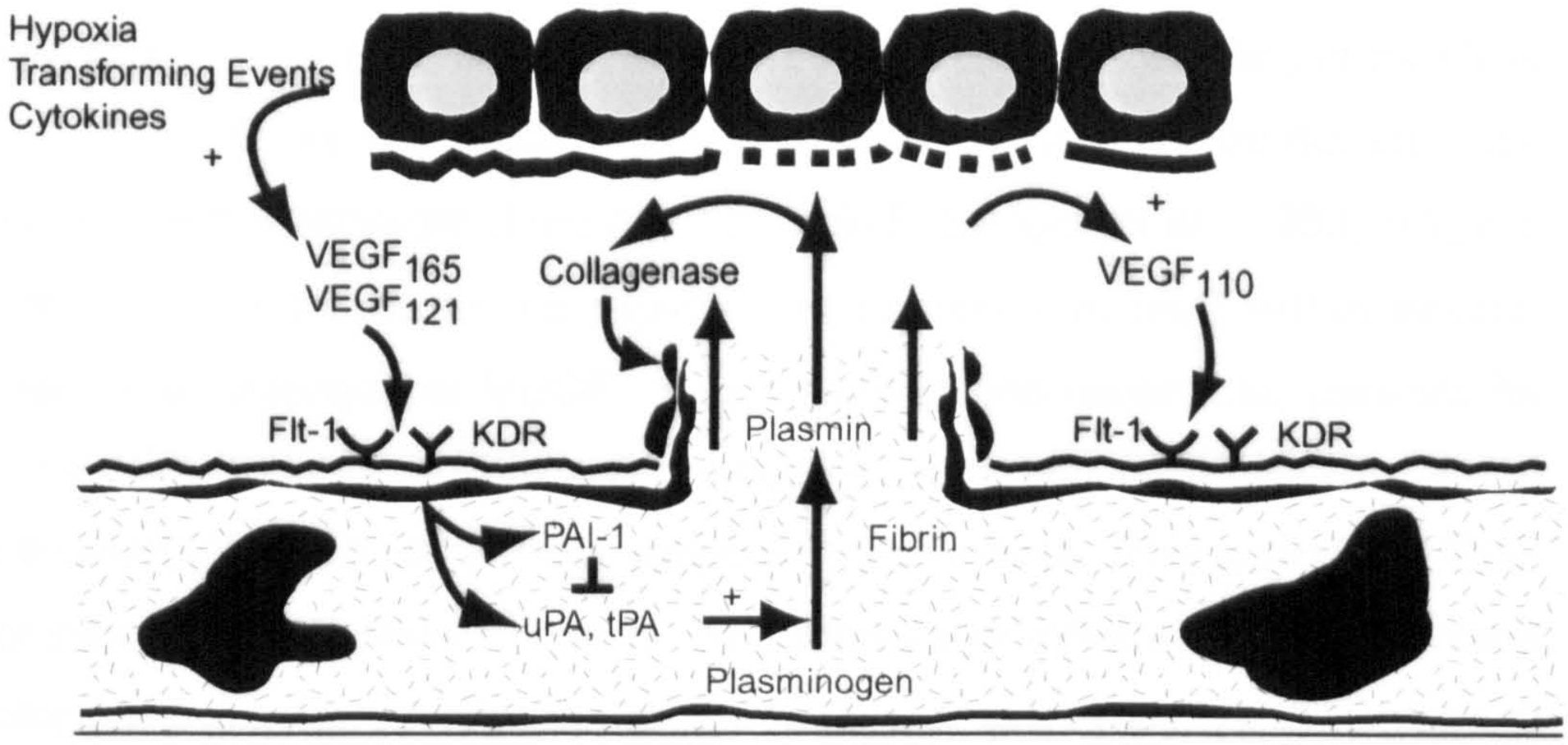


Figure 9.1.2

Schematic representation of the actions of VEGF isoforms on the vascular endothelium. Several stimuli may result in the release of the diffusable alternatively spliced VEGF isoforms ($VEGF_{165}$, $VEGF_{121}$) from a variety of cell types. These proteins may induce a complex series of effects on the vascular endothelium, including cell sprouting, induction of interstitial collagenase, plasminogen activators (PA) and plasminogen activator inhibitor I-1 (PAI-1) as well as extravasation of plasma proteins. Plasminogen activation results in generation of plasmin, which may cleave extracellular matrix-bound VEGF ($VEGF_{189}$, $VEGF_{206}$) to release a diffusable proteolytic fragment ($VEGF_{110}$). Plasmin may also activate procollagenase. Activation of PAI-1 may constitute a negative regulatory step, by inhibiting the action of PA.
(Ferrara & Davis-Symth 1997).

factor (VPF). It one of the most potent vascular permeability agents known, acting at concentrations below 1 nmol/L, with a potency 50,000 times that of histamine (Dvorak et al., 1979, Senger et al., 1983, Collins et al., 1993, Senger et al., 1993 a, b, Dvorak et al., 1995). VEGF increases permeability of a number of vascular beds, including those of the skin, peritoneal wall, mesentery and diaphragm (Dvorak et al., 1979, Senger et al., 1983, Nagy et al., 1995). This effect occurs rapidly and becomes evident within several minutes of an injection of VEGF. It is transient and reversible, persists for less than 30 minutes and is not associated with any detectable injury to endothelial cells or to other microvascular components. The action of VEGF is not blocked by antihistamines or by inhibitors of inflammation that inhibit platelet activating factor (Dvorak et al., 1979, Senger et al., 1983, Collins et al., 1993).

VEGF increases microvascular permeability by enhancing the functional activity of a cytoplasmic organelle called the vesicular-vacuolar organelle (Kohn et al., 1992, Qu-Hong et al., 1994, Dvorak et al., 1995). These are grape - like structures of uncoated vesicles and vacuoles deployed at intervals in the cytoplasm of endothelial cells lining venules and small veins. Vesicular vacuolar organelles interconnect with each other and with endothelial cell plasma membranes by fenestrae that may be open or closed by diaphragms. When the fenestrae are open, macromolecular tracers are able to pass between interconnecting vesicles and vacuoles across the cytoplasm from the vascular lumen to the adluminal basal lamina (Qu - Hong et al., 1995). Thus these organelles provide a pathway whereby plasma and plasma proteins may extravasate from blood vessels into the surrounding extracellular matrix. This extravasation is limited in adult normal tissue but, in tumour vessels and normal skin following local injection of VEGF, the organelle function is substantially upregulated. It has been proposed that an increase in microvascular permeability is a crucial step in angiogenesis associated with tumours and wound healing.

<u>Other biological effects of VEGF:</u>

VEGF induces expression of serine protease urokinase - type and tissue -

type plasminogen activators (PA) and also PA inhibitor 1 (PAI -1) in cultured bovine microvascular endothelial cells (Pepper et al., 1991). It also promotes expression of urokinase receptor (uPAR) in vascular endothelial cells (Mandriota et al., 1995) and of metalloproteinase interstitial collagenase in human umbilical vein endothelial cells (Unemori et al., 1992). This interaction of uPA and uPAR is an important element in the chain of cellular processes that mediate cellular invasion and tissue remodeling (Mignatti et al., 1989, Pepper et al., 1990). The co-induction of PA and collagenase by VEGF facilitates migration and sprouting of endothelial cells. These findings are suggestive of a proangiogenic property of VEGF and are in accord with the hypothesis that extracellular proteolysis, appropriately balanced by protease inhibitors, is required for normal capillary morphogenesis. VEGF also stimulates the release of von Willebrand factor from endothelial cells (Brock et al., 1991).

An additional effect of VEGF on vascular endothelium is stimulation of hexose transport (Pekala et al., 1990). This action may have relevance for increased energy demands during endothelial cell proliferation or inflammation.

VEGF has a promoter effect on monocyte chemotaxis (Clauss et al., 1990) and regulates natural killer cell adhesion to tumour endothelium (Sasaki et al., 1991, Melder et al., 1996). VEGF was also shown to induce colony formation by mature subsets of granulocyte - macrophage progenitor cells previously stimulated with a colony -stimulating factor (Broxmeyer et al., 1995). The common origin of endothelial and haematopoietic cells and the presence of VEGF receptors in progenitor cells may explain these findings as early as haemangioblasts, in blood islands in the yolk sac.

VEGF induces dose dependent vasodilatation in-vitro (Ku et al., 1993) and, when injected intravenously in experimental rats, produces transient tachycardia, hypotension and a decrease in cardiac output (Yang et al., 1996). Such effects probably result from a decrease in venous return mediated primarily by endothelial cell derived nitric oxide. These

haemodynamic effects are not unique to VEGF because angiogenic factors such as aFBF and bFBF may also induce nitric oxide mediated vasodilatation and hypotension (Cuevas et al., 1996).

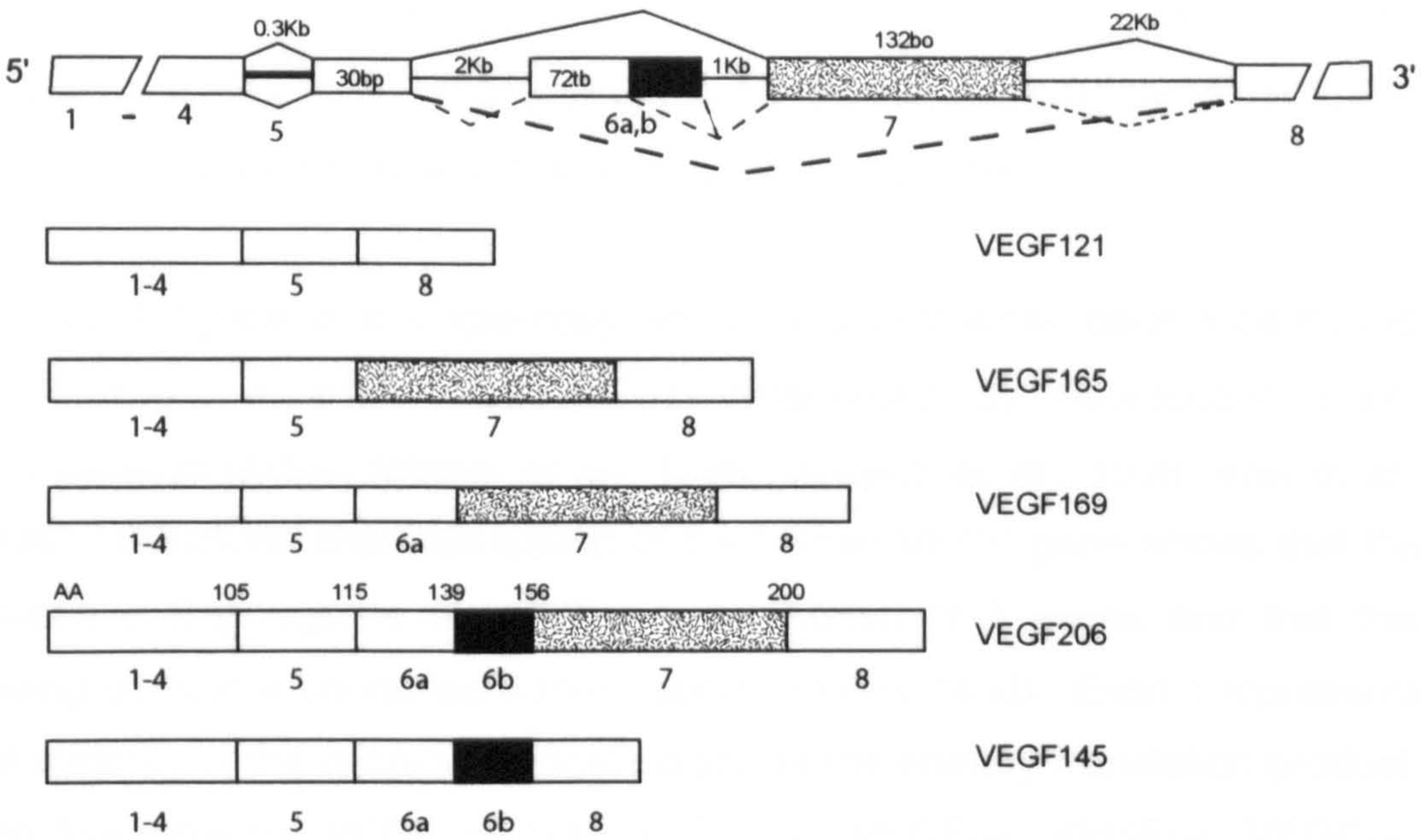
1.2 Molecular characterisation of VEGF and the VEGF gene:

VEGF is a highly conserved, heparin binding, homodimeric disulfide - bonded glycoprotein that has a molecular mass of approximately 46 kilodaltons (kDa). It is inactivated by reducing agents but is heat and acid - stable. Upon reduction, it loses all of its biological activities and separates into major species of 17 to 22 kDa (Senger et al., 1987, Connolly et al., 1989, Ferrara and Henzel, 1989, Gospodarowicz et al., 1989, Conn et al., 1990, Senger et al., 1990, Myoken et al., 1991, Yeo et al., 1991, Ferrara et al., 1992).

The heparin - binding property of VEGF has relevance to measurement of circulating peripheral VEGF concentrations when plasma is used. This is discussed later in the section on Material and Methods.

VEGF was purified from conditioned media of bovine pituitary folliculo-stellate cells, utilising an endothelial cell proliferation assay to monitor the biological activity of the protein (Gospodarowicz et al., 1989, Ferrara and Henzel, 1989). Use of a completely different assay, based on the stimulation of vascular permeability in guinea pig skin, resulted in identification of VPF in tumour ascitic fluid and in conditioned media from several tumour cell lines (Senger et al., 1983, Connolly et al., 1989). VPF was subsequently found to be an angiogenic endothelial cell mitogen and structural characterisation by protein sequencing and cDNA cloning showed it to be identical to VEGF (Connolly et al., 1989, a, b, Keck et al., 1989).

cDNA sequence analysis of a variety of VEGF clones revealed heterogeneity in the VEGF coding region, and indicated the existence of five molecular species (having 121, 145, 165, 189 and 206 amino acids ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$, respectively) of the mature human VEGF subunit (Fig 9.1.3). In addition there exists 164 and 120 residue



bp - basepairs
AA - number of amino acids
1-4, 5, 6a,b, 7 and 8 - exons

Figure 9.1.3

The VEGF gene. Alternate splicing of exons 6 – 8 results in 5 VEGF isoforms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{204}$, $VEGF_{145}$). Exons are represented by boxes and introns by solid lines. The genomic structure of the 5'-end and the 3'-end of the VEGF gene have not been determined, as indicated by the incomplete boxes. The major circulating form is $VEGF_{165}$ which also constitutes the measurable form of VEGF (Houck *et al* 1991).

forms of the mature bovine VEGF subunit (Leung et al., 1989, Keck et al., 1989, Tischer et al., 1989, 1991, Houck et al., 1991). The amino-acid sequence shows 20% homology with platelet derived growth factor (PDGF A and B chains) and 53% with placental growth factor (PlGF).

The VEGF gene is a single-copy and a relatively small gene localised to chromosome 6 p12 - p21.3, as determined by fluorescent in-situ hybridisation (FISH) (Mattei et al., 1996, Vincenti et al., 1996, Wei et al., 1996). Structural characterisation of the human VEGF gene shows that the protein coding regions of VEGF are composed of 8 exons and that this coding domain is contained within`` approximately 14 kb. Exon 1 represents the majority of the secretion signal portion of the primary translation product. The five different VEGF mRNA's ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) arise from alternative splicing of the VEGF gene. A cDNA clone encoding $VEGF_{189}$ was first isolated from U-937 cells (Keck et al., 1989). Subsequently cDNA clones encoding $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ were isolated from phorbol ester- activated HL60 cells (Leung et al., 1989). RNA species corresponding to all forms have been now been detected in cultured human fetal vascular smooth muscle (VSM) cells via cDNA cloning and polymerase chain reaction (PCR).

The alternative exon splicing involves the last four exons. In all species the first four exons are conserved. Mature $VEGF_{189}$ retains coding sequences from exons 2-8. If exon 6 is removed, VEGF $_{165}$ is generated; if exon 7 is removed $VEGF_{145}$ is generated and if both exons 6 and 7 are removed, $VEGF_{121}$ is generated. $VEGF_{121}$, therefore is encoded by exons 1-5 and 8. The most abundant clone, $VEGF_{165}$, encodes a 165 amino acid polypeptide after cleavage of a 26 amino acid hydrophobic signal sequence.

$VEGF_{206}$ has an additional insertion of 17 amino acids. There is no intron, however, between the coding sequence of the 24 amino acid insertion of $VEGF_{189}$ and the additional 17 amino acid insertion found in $VEGF_{206}$ (Fig 9.1.3).

The domain encoded by exons 1-5 contains information required for the recognition of the known VEGF receptors, (KDR/Flk-1 and Flt-1) and is present in all isoforms. The peptide encoded by exon 7 also seems to affect receptor recognition patterns of VEGF isoforms.

Although $VEGF_{145}$ variant has been detected in reverse PCR experiments from placental tissue, the protein encoded by this mRNA has not yet been characterised (Charnock-Jones et al., 1993, Cheung et al., 1995). It was later found that this variant was the major VEGF mRNA expressed in several cell lines derived from carcinomas of the female reproductive tract (Poltorak et al., 1997). Recombinant $VEGF_{145}$ induced proliferation of vascular endothelial cells and induced angiogenesis in vivo. Like $VEGF_{165}$, $VEGF_{145}$ binds to heparin as effectively and binds to KDR/Flk-1 receptor of endothelial cells but not to Flt-1, unlike $VEGF_{165}$. Basic FGF-depleted extra cellular matrix containing bound $VEGF_{145}$ induced proliferation of endothelial cells, indicating that bound $VEGF_{145}$ is active. $VEGF_{145}$ therefore seems to possess a unique combination of biological properties distinct from other species.

1.3 Properties of VEGF isoforms:

The shortest isoform of VEGF, $VEGF_{121}$, is a weakly acidic, 34-36 kDa, homodimeric polypeptide. It is secreted and freely soluble in conditioned medium of transfected cells and does not bind to heparin. $VEGF_{165}$, the most common form of VEGF, is a homodimeric protein of approximately 45 kDa. The 44 amino acids in the carboxyl terminal of VEGF, which are in addition compared with $VEGF_{121}$, convert it to a heparin binding, basic polypeptide. $VEGF_{165}$ is secreted, but 50-70% binds to cell surface or extracellular matrix (ECM). It is $VEGF_{165}$, which comprises the measurable form of circulating VEGF by commercially available enzyme immunoassay kits, described in the section on Material and Methods. $VEGF_{189}$, which has an additional 24 amino acids, is highly enriched in basic residues and $VEGF_{206}$, which has an additional 17 amino acids compared with $VEGF_{189}$, binds much more tightly to heparin. Little or no $VEGF_{189}$ is found in freely soluble forms (Houck et al., 1992, Ferrara, 1992). $VEGF_{206}$ and $VEGF_{189}$ are

almost completely sequestrated in ECM and released from the bound state by a variety of agents such as suramin, heparin or heparinase, suggesting that their binding site is represented by proteoglycans containing heparin like moieties (Houck et al., 1992, Park et al., 1993). Previous studies have demonstrated that differences in isoelectric point and affinity for heparin may profoundly affect the bioavailabilty of VEGF (Houck et al., 1992, Park et al., 1993). $VEGF_{165}$ produced a dose dependent stimulation of growth of bovine capillary endothelial cells. This effect was duplicated by $VEGF_{121}$. Neither $VEGF_{189}$ nor $VEGF_{206}$ however, displayed significant mitogenic activity. Binding of the long forms of VEGF to heparan sulfate proteoglycans in the ECM could provide a reservoir of biologically active VEGF available to endothelial cells after its release (Houck et al., 1992). Long forms of VEGF may be released by plasmin after cleavage of the carboxyl (COOH) terminus (Rifkin et al., 1990, Sreenath et al., 1992, Keyt et al., 1996). This action generates a bioactive proteolytic fragment with a molecular mass of 34 kDa. Plasminogen activation and generation of plasmin have been shown to play an important role in the angiogenesis cascade. Thus, proteolysis of VEGF is likely to occur in vivo. This may be important in the microenvironment of a tumour where increased expression of protease, including PA, is well documented (Rifkin et al., 1990, Sreenath et al., 1992).

When various human cDNA libraries were examined by PCR, it was found that a peripheral lymphocytes library predominantly expressed $VEGF_{165}$ while the major transcript in the placental library was $VEGF_{121}$. $VEGF_{189}$ was found in most libraries while $VEGF_{206}$ was exclusively located to fetal liver library (Houck et al., 1991).

1.4 Regulation of VEGF gene expression:

Numerous mechanisms regulate VEGF gene expression, such as hypoxia, cytokines and cell differentiation and transformation.

A. Hypoxia:

Tissue oxygen tension (pO_2) plays a major role in VEGF gene expression, both in-vitro and in vivo. VEGF gene expression is rapidly and reversibly induced by exposure to low pO_2 in a variety of normal and transformed cultured cell types. Studies have also shown VEGF mRNA to be highly expressed in the microenvironment of a tumour and in the pig myocardium following ischaemic injury (Shweiki et al., 1992, Banai et al., 1994, Brogi et al., 1994, Minchenko et al., 1994, Shima et al., 1995).

B. Cytokines:

Several cytokines and growth factors up-regulate VEGF mRNA expression and induce release of VEGF.

Exposure of human keratinocytes to EGF, TGF-alpha and TGF-beta resulted in up-regulation of VEGF gene expression (Detmar et al., 1994, Detmar et al., 1995, Frank et al., 1995). IGF-1 induced VEGF mRNA and protein in cultured rectal carcinoma cells (Warren et al., 1992). Therefore, in addition to its mitogenic effect on malignant cells, IGF - 1 may also facilitate tumour growth via an increase in the vascular supply, mediated by VEGF. It has been proposed that VEGF may function as a paracrine mediator for indirectly acting angiogenic agents such as TGF-beta (Petrovaara et al., 1994). Furthermore, IL-6, IL-1 beta, IL-1 alpha and PGE_2, have also been shown to induce VEGF mRNA expression in various cell lines, aortic smooth muscle cells and cultured synovial fibroblasts respectively (Li et al., 1995, Ben-Av et al., 1995, Cohen et al., 1996). IL-6 expression is elevated in tissues that undergo active angiogenesis. IL-6 itself does not induce proliferation of endothelial cells. It does however, indirectly induce angiogenesis by inducing VEGF expression.

C. Cell differentiation and transformation.

Cell differentiation (Claffey et al., 1992) and specific transforming events (Kieser et al., 1994) have been shown to play an important role in the regulation of VEGF gene expression. Unrelated alteration in cellular regulatory pathways also results in VEGF up-regulation. This event, therefore, may be a final common pathway necessary for uncontrolled proliferation in vivo.

1.5 The VEGF receptors:

A: VEGF binding sites:

Two high-affinity VEGF binding sites were initially described on the surface of bovine endothelial cells. These had dissociation constants (K_d) of 10 pM and 100 pM and molecular masses in the range of 180-200 kDa (Plouet et al., 1990, Vaisman et al., 1990). Lower affinity binding sites, involved in mediating chemotactic effects of monocytes by VEGF, were later described (Shen et al., 1993). Recently, the presence of low affinity, low molecular mass (120 -130 kDa), receptors on certain tumour and endothelial cells has been described (Gitay-Goren et al., 1996, Soker et al., 1996).

Ligand autoradiography studies on fetal and adult rat tissue sections demonstrated high-affinity VEGF binding sites on vascular endothelium of large and small vessels (Jakeman et al., 1992, 1993). These VEGF binding sites were also observed in the haemangioblasts in the blood islands of the yolk sac, suggesting that expression of VEGF receptors is one of the earliest events in endothelial cell expression (Jakeman et al., 1993).

B. The receptors:

Two homologous VEGF receptors, Flt -1 (fms-like tyrosine kinase) and KDR (kinase domain region), have been identified which bind VEGF with high affinity (Fig 9.1.4). The amino - acid sequences of Flt-1 and KDR share 45% identity. Each receptor is 1300 amino-acid residues long, is composed of 7 immunoglobulin (Ig) -like domains in the extracellular domain (ECD) and contains the ligand binding region. It has a single, short, membrane - spanning region and a region containing tyrosine kinase domains that are interrupted by a kinase insert domain. The murine homologue of KDR is Flk-1 (fetal liver kinase-1). It shares 85% sequence identity with human KDR (Shibuya et al., 1990, Terman et al., 1991, Matthews et al., 1991, de Vries et al., 1992, Terman et al., 1992, Millauer et al., 1993, Quinn et al., 1993, Thomas et al., 1996). Flt -1 has a higher affinity for human recombinant $VEGF_{165}$ and PlGF compared with KDR/Flk-1, which has a lower affinity for human recombinant $VEGF_{165}$ and none for PlGF.

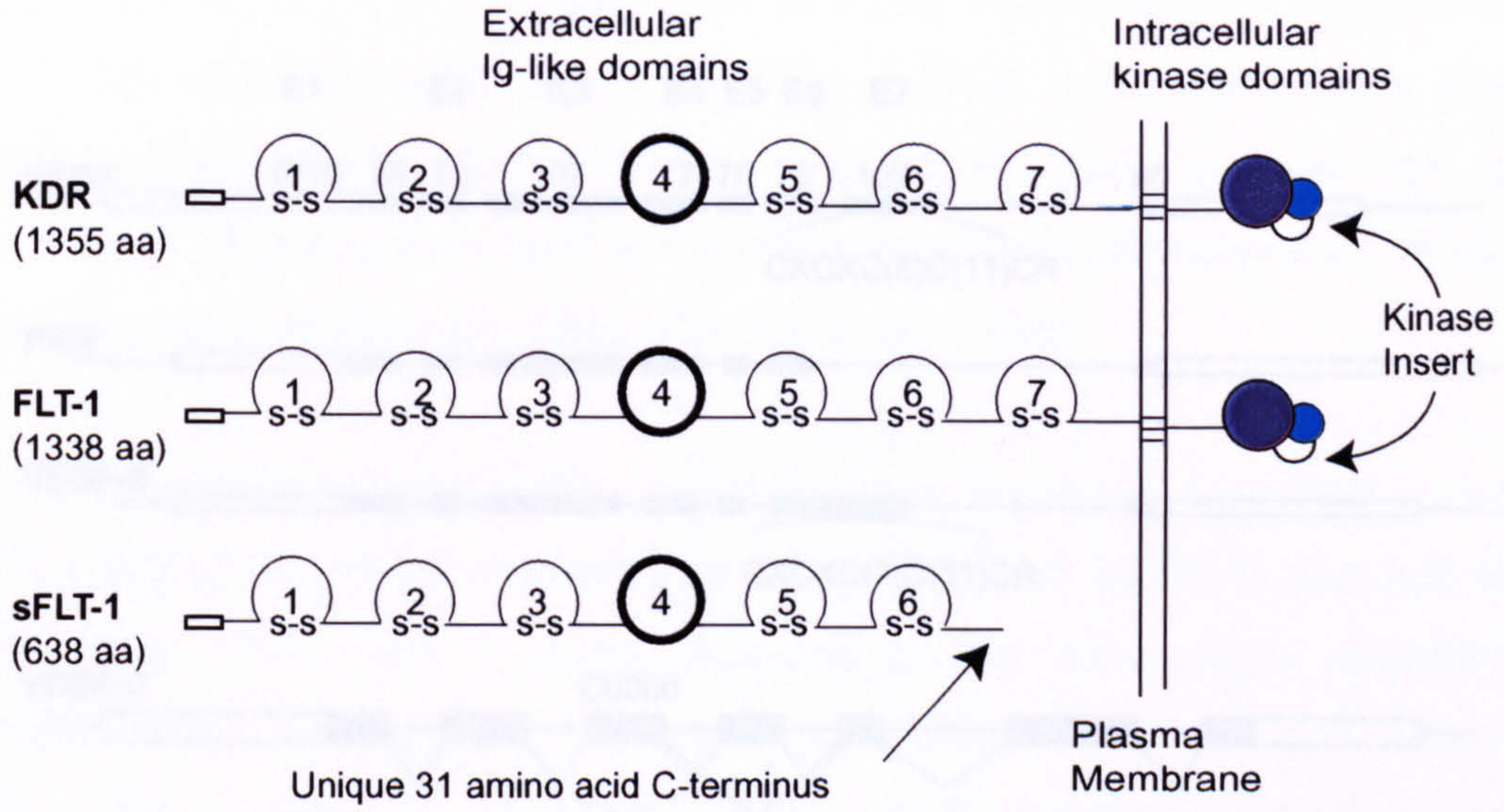


Figure 9.1.4

The structure of VEGF receptors, KDR, FLT-1 and SFLT-1.
The full-length VEGF specific KDR receptor and the homologous Flt-1 receptor, which binds both VEGF and PIGF are each composed of 7 extracellular Ig-like domains containing the ligand-binding region, a single plasma membrane spanning sequence and intracellular tyrosine kinase domains containing a kinase insert sequence. An alternatively expressed soluble truncated form of Flt-1, denoted sFlt-1, containing the N-terminal 6 Ig-like domains followed by a unique 31-amino acid residue C-terminal sequence functions as an inhibitor of VEGF mitogenic activity.
Human amino acid (aa) residue sequence lengths are given in parentheses (Thomas *et al* 1996).

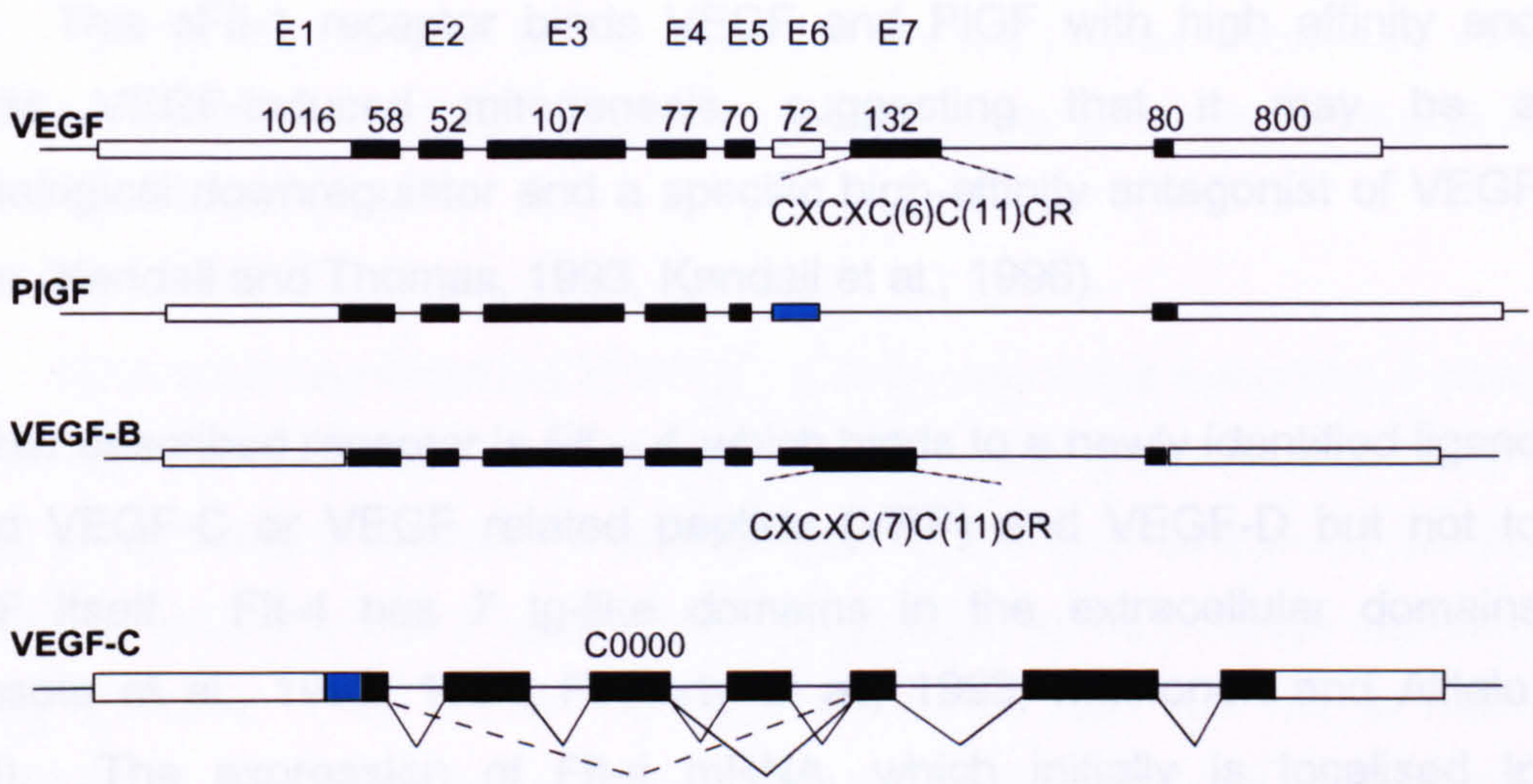


Figure 9.1.5

VEGF and VEGF related molecules. The structure of VEGF is compared with VEGF related molecules eg PIGF, VEGF-B, VEGF-C. CXCXC and C0000 denote cysteine-rich motifs typical of a silk protein.
(Chilov *et al* 1997)

An alternatively spliced recombinant soluble form of Flt-1 *(sFlt-1),* which lacks the seventh Ig-like domain, transmembrane sequence and the cytoplasmic domain, has been identified in human umbilical vein endothelial cells. This sFlt-1 receptor binds VEGF and PlGF with high affinity and inhibits VEGF-induced mitogenesis, suggesting that it may be a physiological downregulator and a specific high-affinity antagonist of VEGF action (Kendall and Thomas, 1993, Kendall et al., 1996).

Another described receptor is *Flt – 4*, which binds to a newly identified ligand called VEGF-C or VEGF related peptide (VRP) and VEGF-D but not to VEGF itself. Flt-4 has 7 Ig-like domains in the extracellular domains (Pajusola et al., 1992, 1994, Finnerty et al., 1993, Mustonen and Alitalo, 1995). The expression of Flt-4 mRNA, which initially is localised to angioblasts and venules in the early embryo, becomes restricted to lymphatic endothelium at later stages of development. This expression pattern may suggest a role of Flt-4 in the regulation of lymphangenesis (Kaipainanen et al., 1995).

Recently a new receptor, *neuropilin*, has been identified, which specifically binds to $VEGF_{165}$ (Soker et al., 1997).

C. Signal transduction:

Signal transduction of VEGF receptors is induced by phosphorylation of various proteins. Studies indicated that Flt-1 and KDR/Flk-1 have different signal transduction properties. KDR/Flk-1 undergoes a strong ligand-dependent tyrosine phosphorylation in intact cells, while Flt-1 has a weak or an undetectable response (Waltenberger et al., 1994, Seetharam et al., 1995). PlGF, which binds with high affinity to Flt-1 but not to KDR / Flk-1 and lacks direct mitogenic or permeability enhancing properties, does not stimulate tyrosine phosphorylation in endothelial cells. Therefore, interaction with KDR / Flk-1 seems to be a critical requirement to induce the full spectrum of VEGF biological responses.

1.6 VEGF- related molecules:

Three VEGF related genes have recently been identified from mammalian sources. The encoded factors are PlGF, VEGF-B and VEGF-C/VRP (Fig 9.1.5). The biological roles of these factors are still unclear, however, their structural homology to VEGF suggests they may play a role in regulation of blood vessel growth.

PlGF: Soon after identification of VEGF, a DNA sequence encoding a close homologue was reported. It was denoted placental growth factor (PlGF) based on its original source. It shares 54% amino-acid sequence identity with VEGF, including the 8 conserved Cys residues in the putative receptor-binding domain. PlGF binds with high affinity to Flt-1 but not to KDR. Purified PlGF demonstrated minimal vascular endothelial cell growth and permeability activity, suggesting that binding with KDR receptor is essential for both activities.

VEGF-C/ VRP:

VEGF-C/VRP is a secreted protein with 399 amino acid residues and has a 32% identity to VEGF. Its COOH - terminal contains a 180 amino-acid region, not found in VEGF. VEGF-C/VRP stimulates growth of human lung endothelial cells, but at a 100 -fold lower potency than $VEGF_{165}$ (Lee et al., 1996). VRP mRNA is found in several human tissues including adult heart, placenta, ovary, small intestine and in the fetal lung and kidney. VEGF-C binds to receptors KDR and Flt-4.

VEGF- B:

VEGF-B is a member of the VEGF gene family, which stimulates human and bovine vascular endothelial cells. The molecule, including the signal peptide, consists of 188 amino acids. It is mainly distributed in skeletal muscle and myocardium and is co-expressed with VEGF. VEGF- B is expressed as a membrane bound protein, similar to the longer forms of VEGF, which can be released in a soluble form after addition of heparin. It is also able to form heterodimers with VEGF thus leading to the hypothesis that it may regulate angiogenesis, particularly in the muscle (Olofsson et al.,

1996, Grimmond et al., 1996). The receptor for VEGF-B has not yet been described.

VEGF- D:

This novel protein which shares structural homology with VEGF was recently described (Yamada et al., 1997). The receptors for VEGF-D are KDR and Flt-4 (Achen et al., 1998).

1.7 Other angiogenic, vasoactive and haemostatic factors:

These factors are briefly mentioned here since they may act in conjunction with VEGF to promote angiogenesis.

PDGF: VEGF is distantly related to both the A and B chains of PDGF. PDGF-A is similar to $VEGF_{121}$ and $VEGF_{165}$, which lack the exon 6 amino acids. PDGF is mitogenic to human endometrial stromal cells and is known to enhance the effect of EGF on these cells (Chegini et al., 1992).

Endothelin:

Endothelins are potent vasoconstrictors derived from endothelial cells. Specific binding sites for endothelins have been detected in the human endometrium (Davenport et al., 1991). There are data to suggest that endothelin derived from endometrial stroma may act on the adventitial surface of spiral arterioles in the endometrium, promoting the vasoconstriction which is required for menstrual shedding. The endometrium has the capacity to synthesise and degrade endothelin on a cyclical basis, consistent with its role as a vasoactive peptide during menstruation (Casey et al., 1991).

Basic fibroblast growth factor (bFGF):

bFGF is an 18,000 Dalton, heparin binding angiogenic protein that is highly mitogenic for capillary endothelial cells and can induce angiogenesis in vivo (Cordon-Cardo et al., 1990).

It has been shown by immunohistochemistry, to be present in the basement

membrane of medium sized blood vessels, smooth muscle cells of the tunica media and capillary endothelial cells and the basal lamina of most tissues examined. Recent studies have suggested that VEGF and bFGF may have synergistic effects on the induction of angiogenesis in-vitro but bFGF does not increase vascular permeability.

Nitric Oxide:

Nitric oxide (NO) or endothelium derived relaxing factor (EDRF), released by vascular endothelial cells, is a potent vasodilator, perhaps more important than prostacyclin in maintaining vascular tone. It has been postulated that NO along with cytokines may mediate VEGF mRNA expression.

Epidermal growth factor (EGF):

EGF is a potent mitogen. EGF enhances uterine epithelial cell growth in-vitro and can replace oestrogen in stimulation of the female genital tract growth and differentiation in vivo (Guidice et al., 1994). In humans, EGF mRNA and peptide are present in the endometrium but its concentrations do not change during the menstrual cycle (Murphy et al., 1991).

Insulin like growth factors (IGF):

Insulin like growth factors (IGF-I and IGF-II) promote cellular mitosis and differentiation. They circulate bound to a family of binding proteins (IGFBP's) that also regulate IGF action on target tissues (Guidice, 1992). The IGF's are believed to promote endometrial cellular mitosis and differentiation during the menstrual cycle and early pregnancy (Guidice et al., 1993).

Transforming growth factor - Beta

TGF- beta is a multifunctional peptide that stimulates as well as inhibits cellular proliferation and differentiation in a variety of cells, acting via autocrine or paracrine mechanisms. It has been shown to up-regulate transcription of both bFGF and VEGF in vascular smooth muscle cells. It therefore mainly acts as an indirect cytokine (Asahara et al., 1995).

Tumour necrosis factor - (TNF-alpha):

VEGF has been shown to enhance the activity of this inflammatory mediated TNF in the regulation of the expression of at least two endothelial cell proteins. VEGF and TNF stimulate tissue factor expression by endothelial cells and the two cytokines synergistically act to initiate the pro-coagulant response (Connolly, 1991). TNF however, completely blocks the mitogenic activity of VEGF. This suggests that even though the activities of these factors overlap the overall spectrum of activities of VEGF and TNF are distinct (Connolly, 1991).

Platelet derived endothelial cell growth factor / thymidine phosphorylase (PD-ECGF/TP):

This non-glycosylated protein was originally isolated from human platelets. PD-ECGF/TP also promotes angiogenesis

1.8 Other inducers and inhibitors of VEGF bioactivity:

Platelet factor-4 (PF4):

The binding of $VEGF_{165}$ but not $VEGF_{121}$, to VEGF receptors on endothelial cells, was shown to be inhibited by angiogenesis inhibitor PF4 (Gengrinovitch et al., 1995). PF4, however, in addition to inhibiting $VEGF_{165}$ induced proliferation of vascular endothelial cells, also inhibited $VEGF_{121}$ induced proliferation of cells, indicating that PF4 can disrupt VEGF receptor mediated signal transduction through an unknown mechanism which does not interfere with $VEGF_{121}$ binding (Gengrinovitch et al., 1995).

Alpha $_2$ Macroglobulin (Alpha$_2$ M):

Alpha $_2$ M is a major serum glycoprotein, which was initially characterised as a protease inhibitor. It is present in serum in high concentrations and binds several proteins such as, insulin, PDGF, TGF-beta and bFGF, through covalent and noncovalent bonds (Soker et al., 1993). It also binds to VEGF, leading to inactivation of the receptor binding ability of VEGF. This binding is inhibited by heparin and heparan sulfate. The inhibitory action of heparin is specific since similar concentrations of polyanionic sugars like dextran sulfate and glycosaminoglycan such as chondroiton sulfate do not inhibit this binding (Soker et al., 1993). Circulating serum VEGF measured with the assays described in this thesis measure total VEGF bound to serum proteins such as alpha 2 macroglobulin.

Lavendustin A and Suramin:

$VEGF_{165}$ induced angiogenesis has been shown to be inhibited by a selective protein kinase (PTK) inhibitor, lavendustin A. Suramin, an inhibitor of heparin binding growth factors, also suppressed the $VEGF_{165}$ elicited neovascular response. This finding suggests that selective inhibition of PTK could have a therapeutic potential in angiogenic diseases where VEGF plays a dominant role (Hu and Fan, 1995).

Angiotensin II (Ang II):

It has also been demonstrated that human vascular smooth muscle cells express abundant VEGF mRNA (Williams et al., 1994) and Ang II potently induced this expression. This Ang II induced vascular permeability factor mRNA expression in vascular smooth muscle cells was inhibited by the specific Ang II receptor antagonist, losartan (Williams et al., 1994).

Section 2: Role of VEGF in the female reproductive system:

In the female reproductive system, the need for additional vasculature is constantly imposed by cyclical evolution of transient structures and by cyclical repair of damaged tissues such as neovascularisation of ovarian follicles and corpora lutea, repair of endometrial vessels and angiogenesis in the embryonic implantation sites. The roles of VEGF in expressing targeted angiogenic responses in theca layers, lutein cells, endometrial stroma and maternal decidua have been described (Shweiki et al., 1993). The same group also demonstrated that VEGF was expressed in 10 different steroidogenic and steroid responsive cell types (theca, cumulus, granulosa, lutein, oviductal epithelium, endometrial stroma, decidua, giant trophoblast cells, adrenal cortex and Leydig cells of the testes) within the reproductive system. Since hormones coordinate cyclical angiogenesis during the menstrual cycle, it has been hypothesised that the factors that mediate this angiogenesis may be directly or indirectly hormone regulated (Fig 9.2.1).

2.1 Neovascularisation of ovarian follicles and corpus luteum (CL):

Using in situ hybridisation (ISH), Shweiki et al., 1993 demonstrated that small preantral follicles did not have a vascular supply of their own. With the onset of follicular growth, capillary networks are acquired within the theca interna and externa layers and the ovarian interstitial tissues. The VEGF produced and secreted by these cells acts on those nearby target cells that express the appropriate receptors. VEGF receptors have been identified on the endothelial cells of large medullary vessels from which thecal vessels originate, on endothelial cells interspersed in the stroma and in capillaries arranged in the peripheral theca layers of the growing follicles (Shweiki et al., 1993). In transplanted murine ovaries, however, VEGF mRNA expression is abundant and predominantly located in cells of the outer ovarian cortex, which normally do not express VEGF (Dissen et al., 1994).

With further growth and maturation of the follicle, the sites of VEGF expression shift to additional ovarian compartments. The first cells, inner to the theca, to express VEGF are the cumulus cells engulfing the oocyte. It is possible that the VEGF in the cumulus oophorus may play a role in

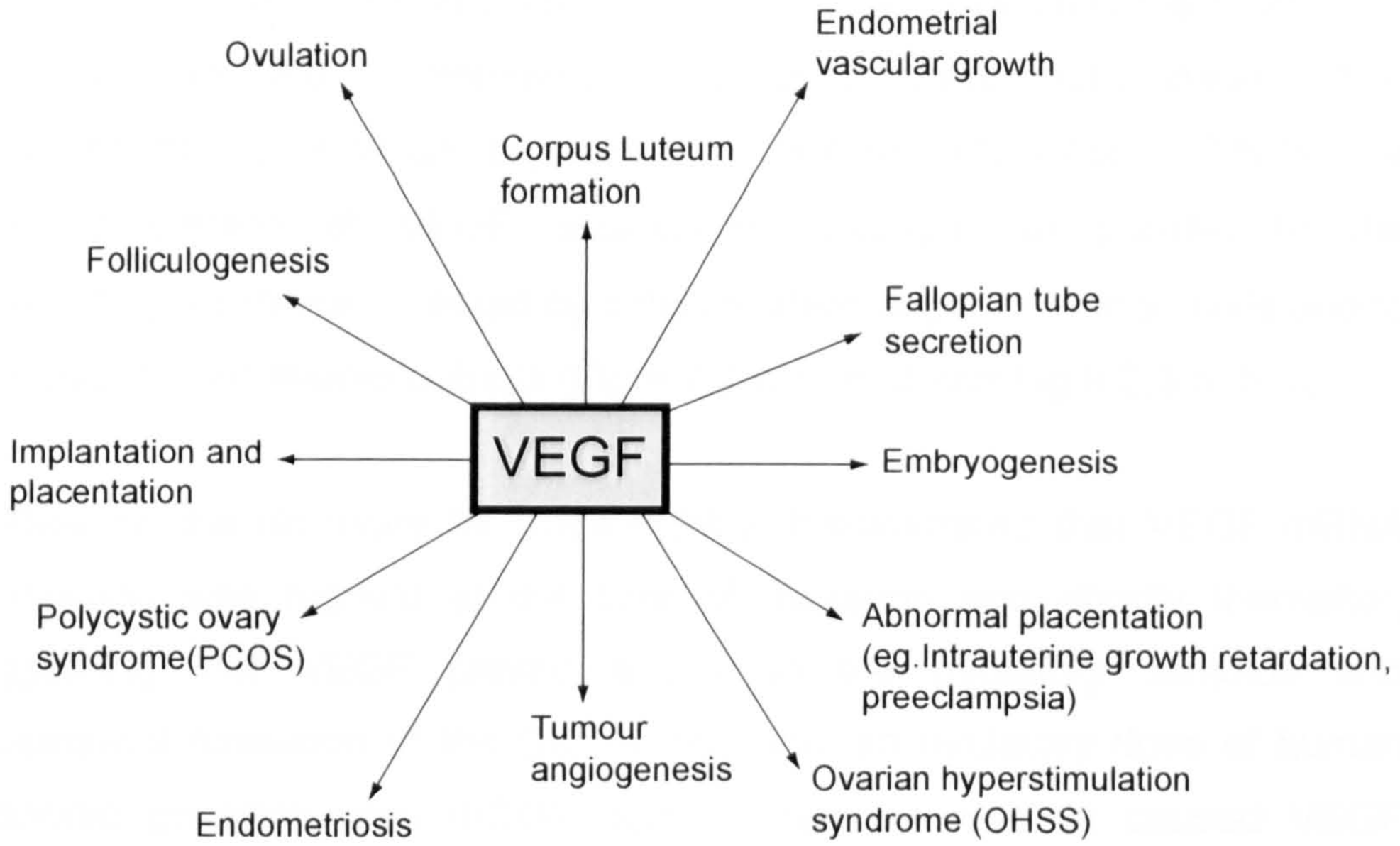


Figure 9.2.1

Role of VEGF in female reproductive system.

fertilisation and subsequent implantation (Phillips et al., 1990). In the granulosa cells, high levels of VEGF are expressed only at the immediate preovulatory stage. Shortly after ovulation, granulosa cells transform into lutein cells and the development of the corpus luteum (CL) ensues. The predominant site of VEGF expression is then the lutein cells. There is a dynamic pattern of VEGF expression, changing in parallel to the gonadotrophin stimuli received by different steroidogenic ovarian cells and to changes in their steroid outputs (Fig 9.2.2 a, b, c, d and Fig 9.2.3 a, b, c).

Studies on the rat ovary by Koos (1995) demonstrated that VEGF mRNA expression was highest at the time of ovulation and shortly thereafter, suggesting that VEGF played a role in the ovulatory stimulus and subsequent formation of the CL. In humans, an ovulatory dose of human chorionic gonadotrophin (hCG), administered by injection caused VEGF mRNA levels and VEGF serum concentrations to rise 8 fold within 4 hours of the injection (Neulen et al., 1995, Christenson and Stouffer, 1997). A recent study by Ansati et al., (1998) demonstrated that VEGF concentrations in the follicular fluid obtained from an immediate preovulatory follicle correlated positively with progesterone concentrations within follicular fluid and with serum LH concentrations (obtained on the same day), providing further evidence of a close relationship of VEGF production with early luteinisation in human follicles during the normal menstrual cycle.

Upon development of the CL, lutein cells proliferate and gradually fill the entire space of the former antrum. Capillaries invade the CL and form an elaborate network whereby each lutein cell resides in close proximity to blood capillaries. The role of VEGF in the development of the CL vasculature is suggested by the abundant production of VEGF by all the lutein cells. Consistent with its role as a paracrine endothelial cell mitogen, it has been shown that CL vasculature possesses VEGF binding activity. To support these findings, Phillips et al., 1990, using in-situ hybridisation analysis have demonstrated that VEGF binding activity was expressed within the endothelial cells comprising the vascular network of the CL, while the ligand was produced by the lutein cells (Ferrara et al., 1992). Lutein cells

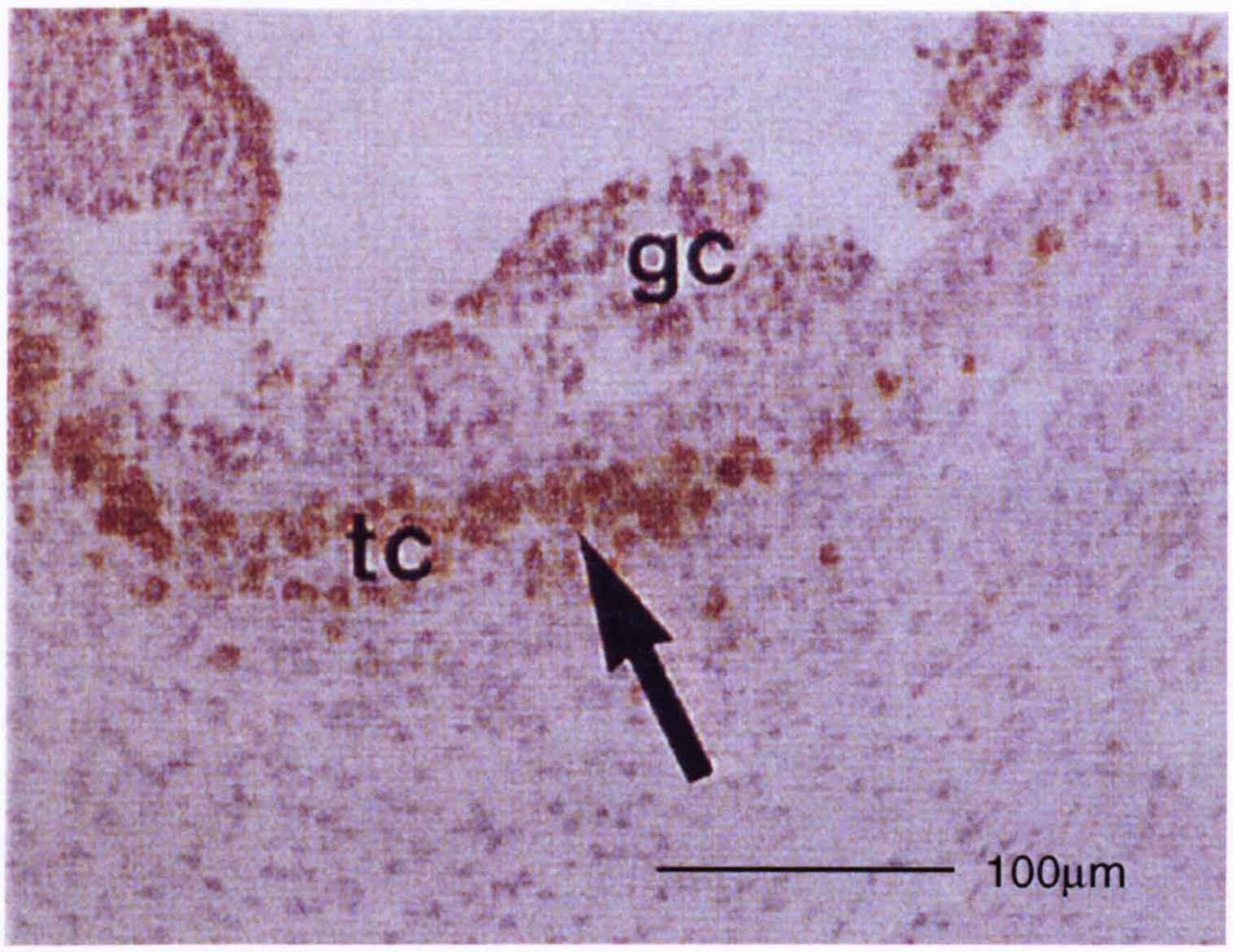


Figure 9.2.2(a)

Immunohistochemical staining for VEGF in a healthy follicle from a human ovary during the early follicular phase. In figures 9.2.2 a-d, cells are stained with rabbit polyclonal antibody to human VEGF and exposed to biotinylated goat antirabbit IgG and then treated with avidin peroxidase complex and 3.5 diamino benzide tetrahydrodionide. Counter stained with haemotoxylin. Arrow points at specific staining for VEGF (brown precipitate). gc- granulosa cell layer, tc-theca cell layer

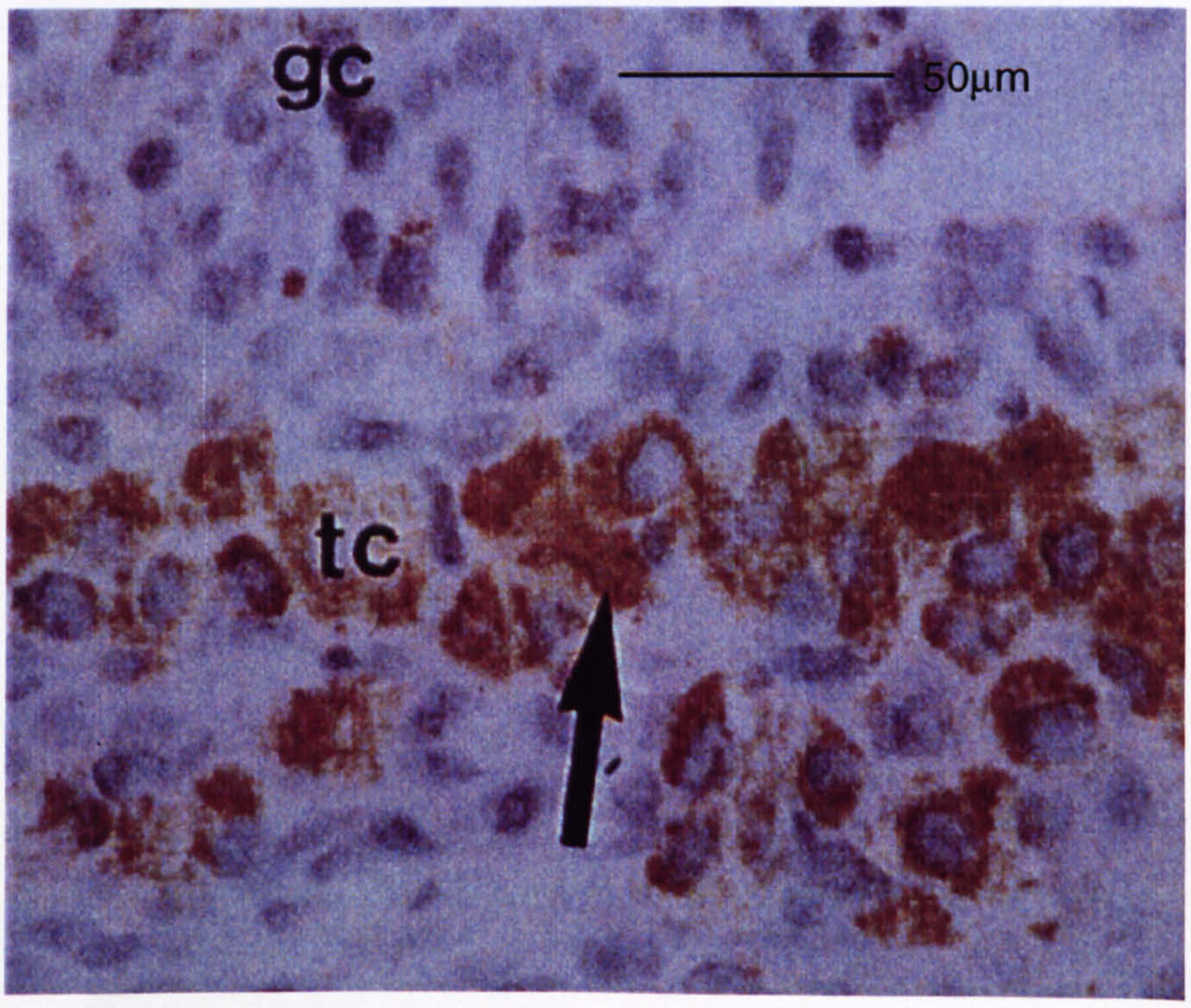


Figure 9.2.2 (b)

Immunohistochemical staining for VEGF in a healthy follicle from a human ovary during the early follicular phase (magnified view). (Figures 9.22 a,b,c,d. adapted from Gordon *et al* 1996).

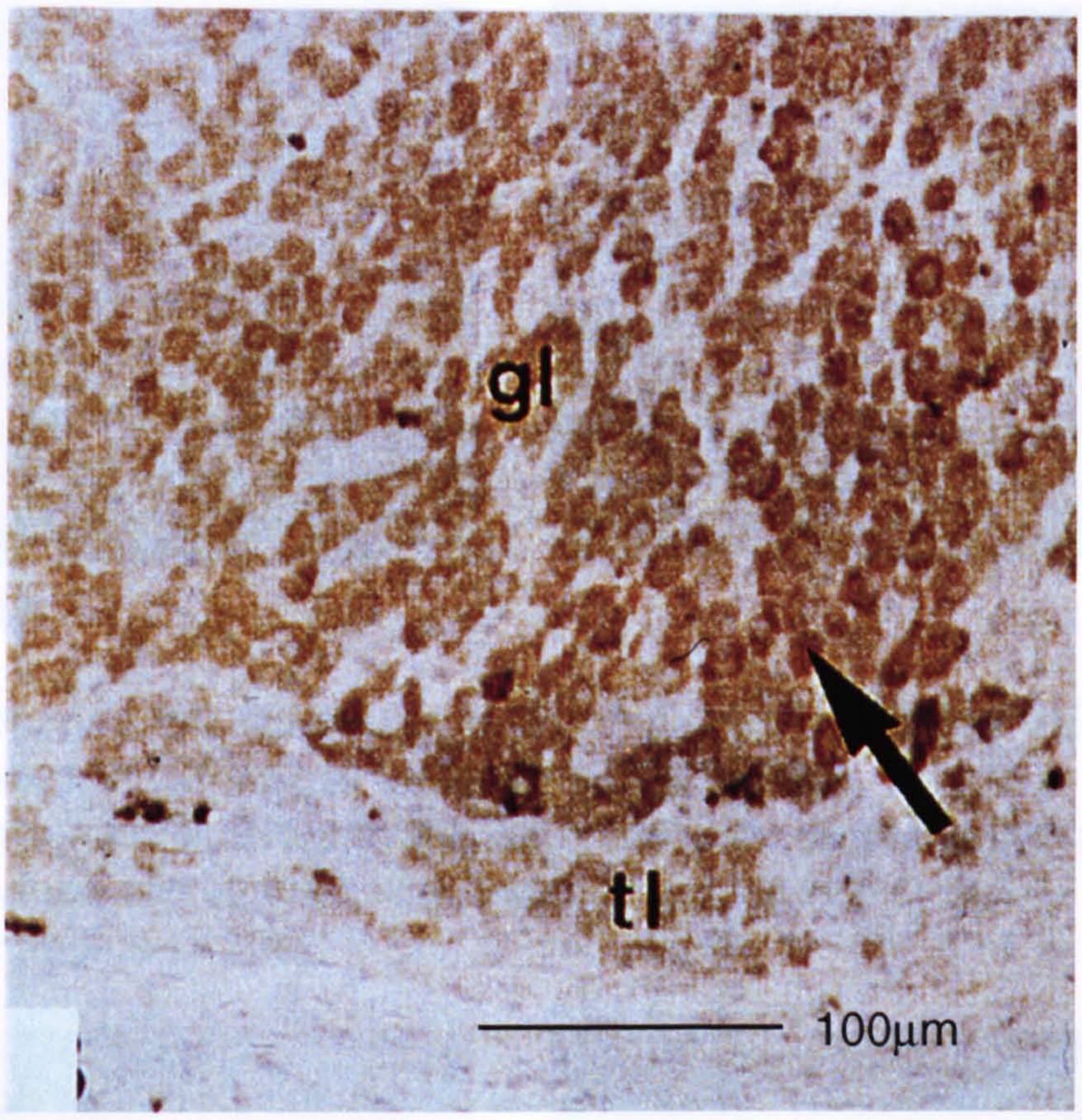


Figure 9.2.2(c)
Immunohistochemical staining for VEGF in a healthy follicle from a human ovary during the midluteal phase. Arrow points at specific staining for VEGF (brown precipitate). gl-granulosa lutein cells, tl - theca lutein cells.

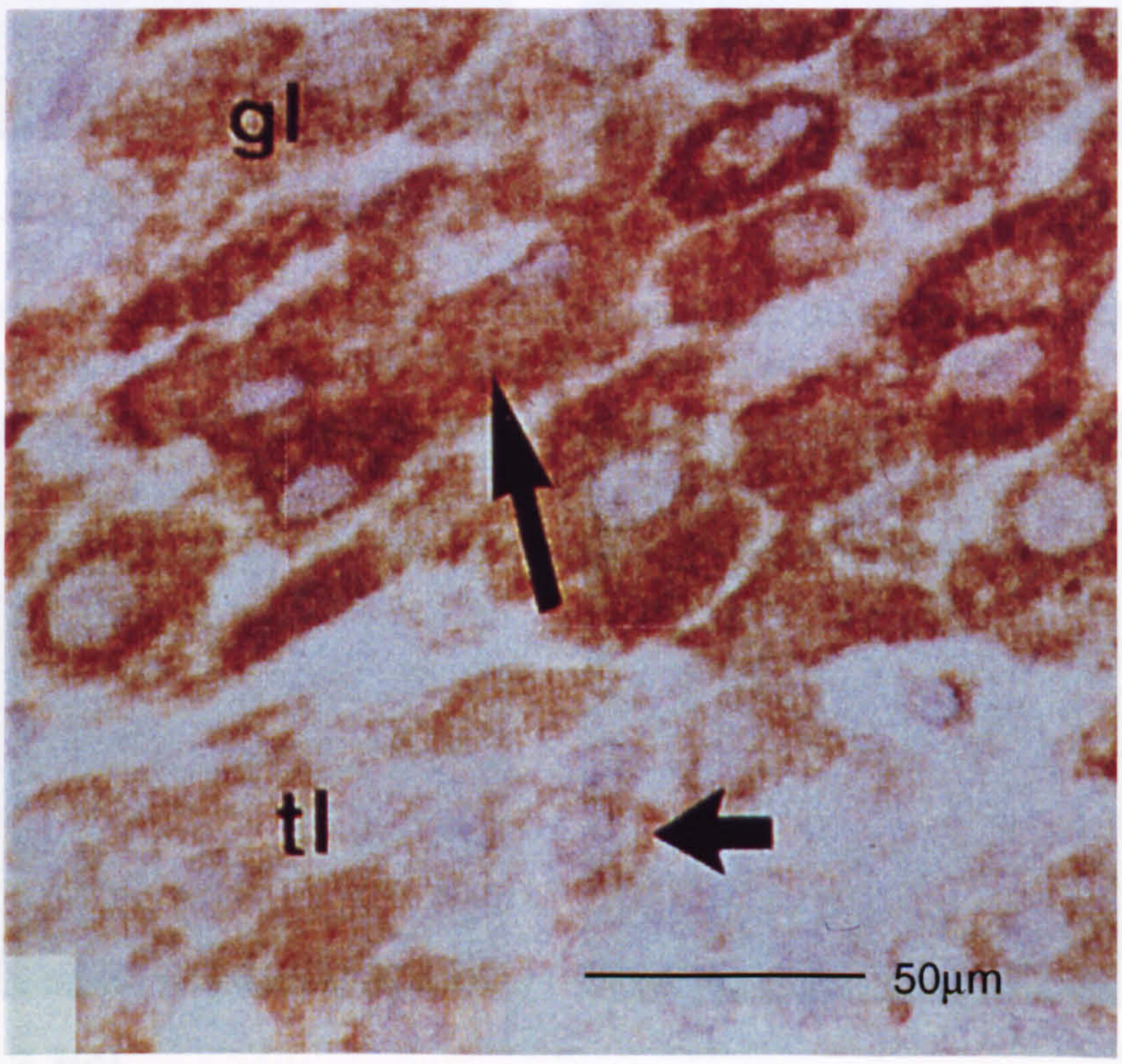


Figure 9.2.2(d)
Immunohistochemical staining for VEGF in a healthy follicle from a human ovary during the midluteal phase (magnified view).

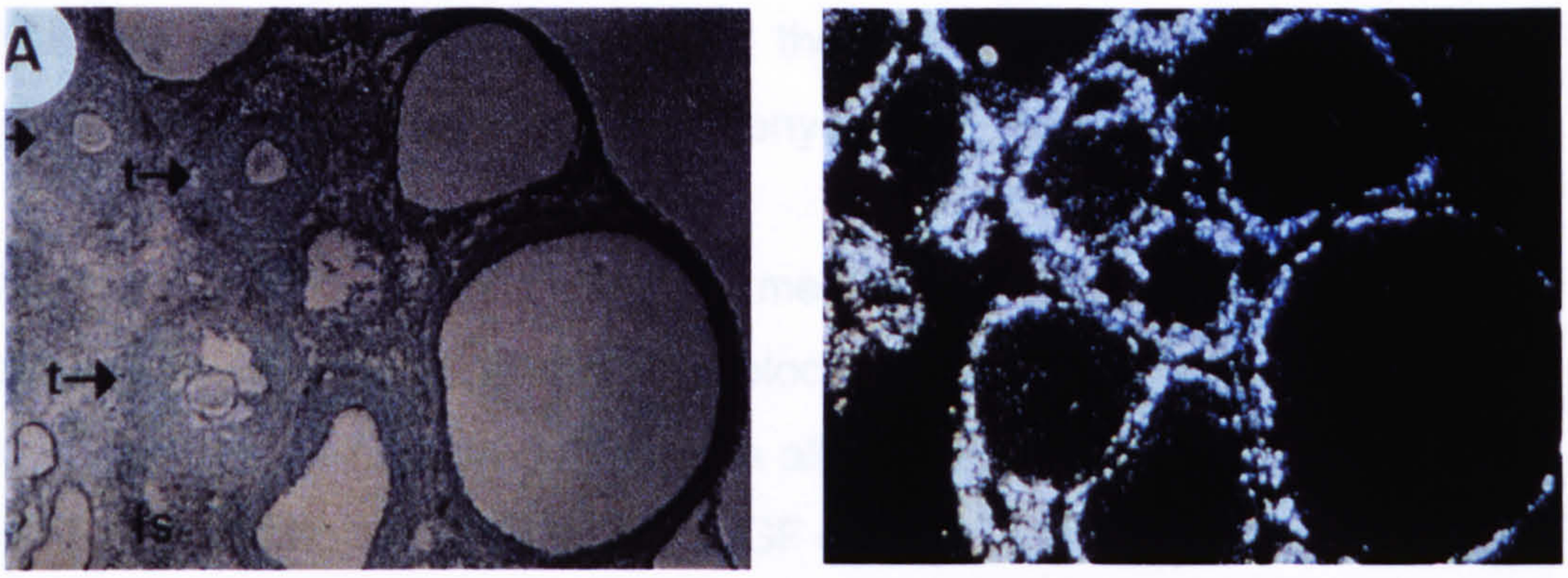


Figure 9.2.3(a)

Right sided images are stained by in situ hybridisation using VEGF specific probe to VEGF to demonstrate VEGF mRNA expression in a murine ovary in the early follicular phase (t-theca cells). Left sided images are stained with haematoxylin eosin stains (all images mangified x220).

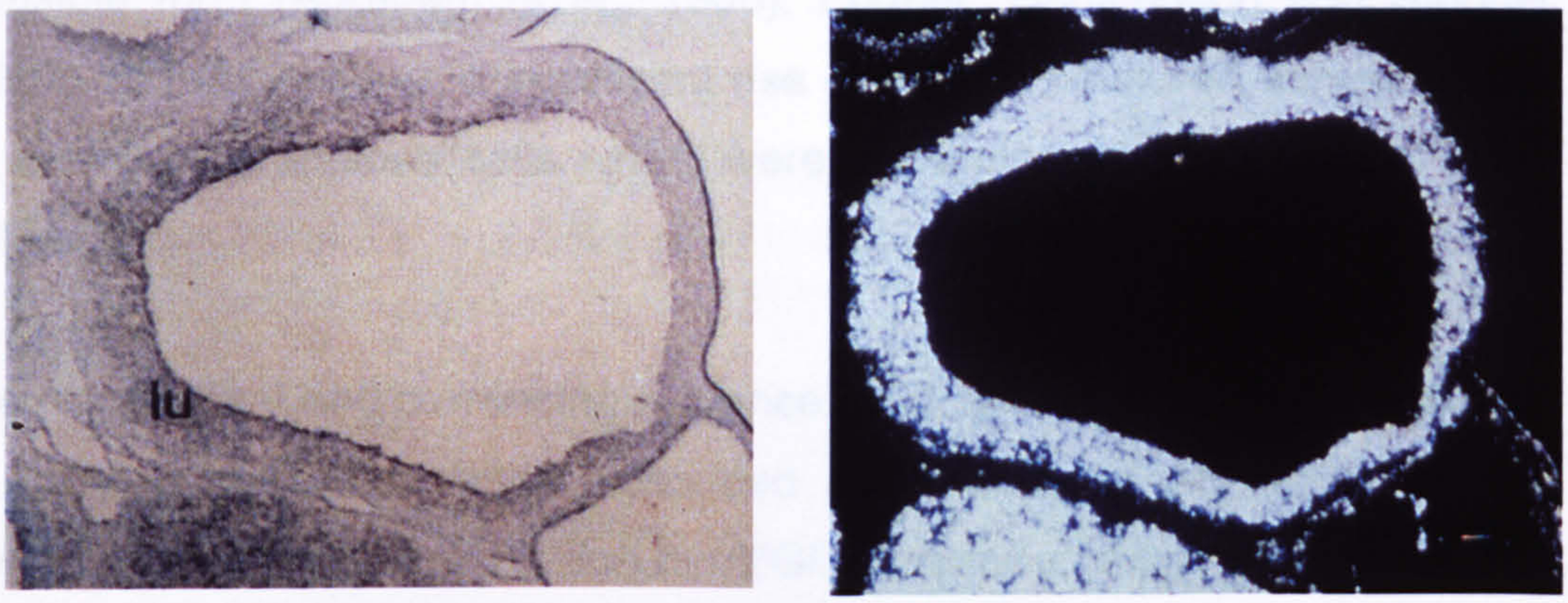


Figure 9.2.3 (b)

In situ hybridisation using VEGF specific probe to demonstrate VEGF mRNA expression in a murine ovary in a preovulatory follicle.

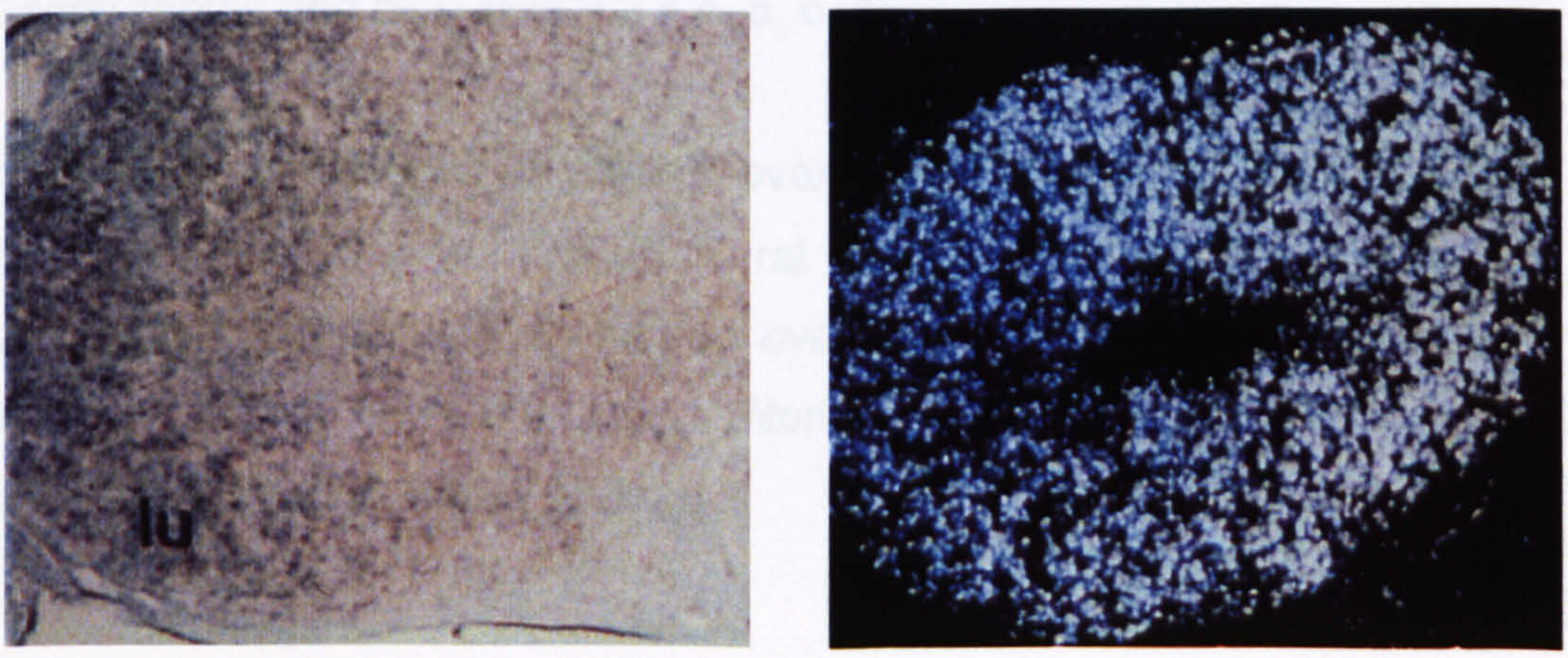


Figure 9.2.3(c)

In situ hybridisation using VEGF specific probe to demonstrate VEGF mRNA expression in a murine ovary in the corpus luteum (lu-lutein cells). (Figures 9.2.3. a-c adapted from Shweiki *et al* 1993)

continue to express VEGF in the fully developed, functional CL. VEGF expression reduces thereafter within the receding corpus luteum at the beginning of menstruation (Fairchild-Benyo et al., 1993).

Although the expression of VEGF messenger RNA is temporally and spatially related to the proliferation of blood vessels in spontaneously cycling and in hormonally induced ovulation in all species, other molecules such as bFGF, angiopoetin 1, 2, PDGF and PlGF are also implicated in corpus luteal angiogenesis. In fact, bFGF was also initially thought to be a major mediator of angiogenesis within the ovary (Gospodarowicz et al., 1987) since it was produced by intact CL (Gospodarowicz et al., 1985) and by cultured granulosa cells (Neufeld et al., 1987). bFGF, however, is an intracellular protein, not significantly released in the cell exterior and secreted in a freely diffusible form (Abraham et al., 1986). Further, Christenson and Stouffer (1997), did not observe a significant rise of bFGF in cultured non-luteinised or luteinised granulosa cells which were stimulated with addition of hCG, FSH or LH.

The most recent and convincing evidence of VEGF as an essential factor for CL angiogenesis has been described by Ferrara et al., 1998. They demonstrated complete inhibition of VEGF bioactivity, suppression of corpus luteal formation and progesterone production by using soluble human and murine tyrosine kinase receptor fixed Fc immunoglobulin (sFlt-1, a VEGF inhibitor), in a murine ovary where ovulation was induced with gonadotrophins and hCG (Fig 9.2.4 a, b, c, d).

Further studies performed on primate ovaries by Ravindranath et al., (1992), and Fairchild-Benyo et al., (1993), on rat ovaries by Yan et al., (1993), and Dissen et al., (1994), and on human ovaries by Kamat et al., (1995) and Yamamoto et al., (1997), using Northern blot analysis and in-situ hybridisation, confirm similar findings.

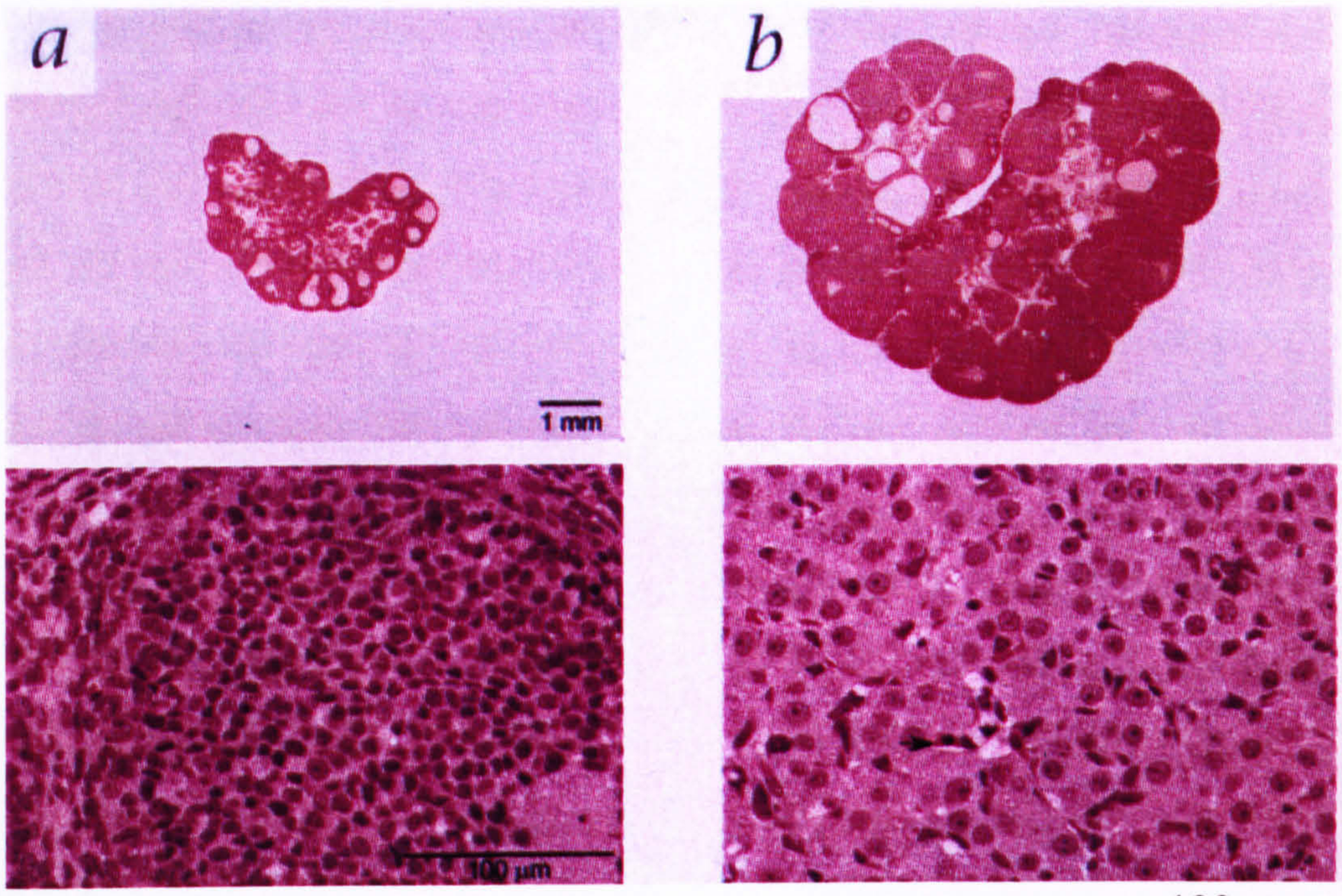


Figure 9.2.4(a,b)

In situ hybridisation using VEGF specific probe. Tissue is counter stained with haemotoxylin. Histological section of a murine ovary. a) ovary unstimulated with gonadotrophins. b) Superovulated with gonadotrophins and hCG.

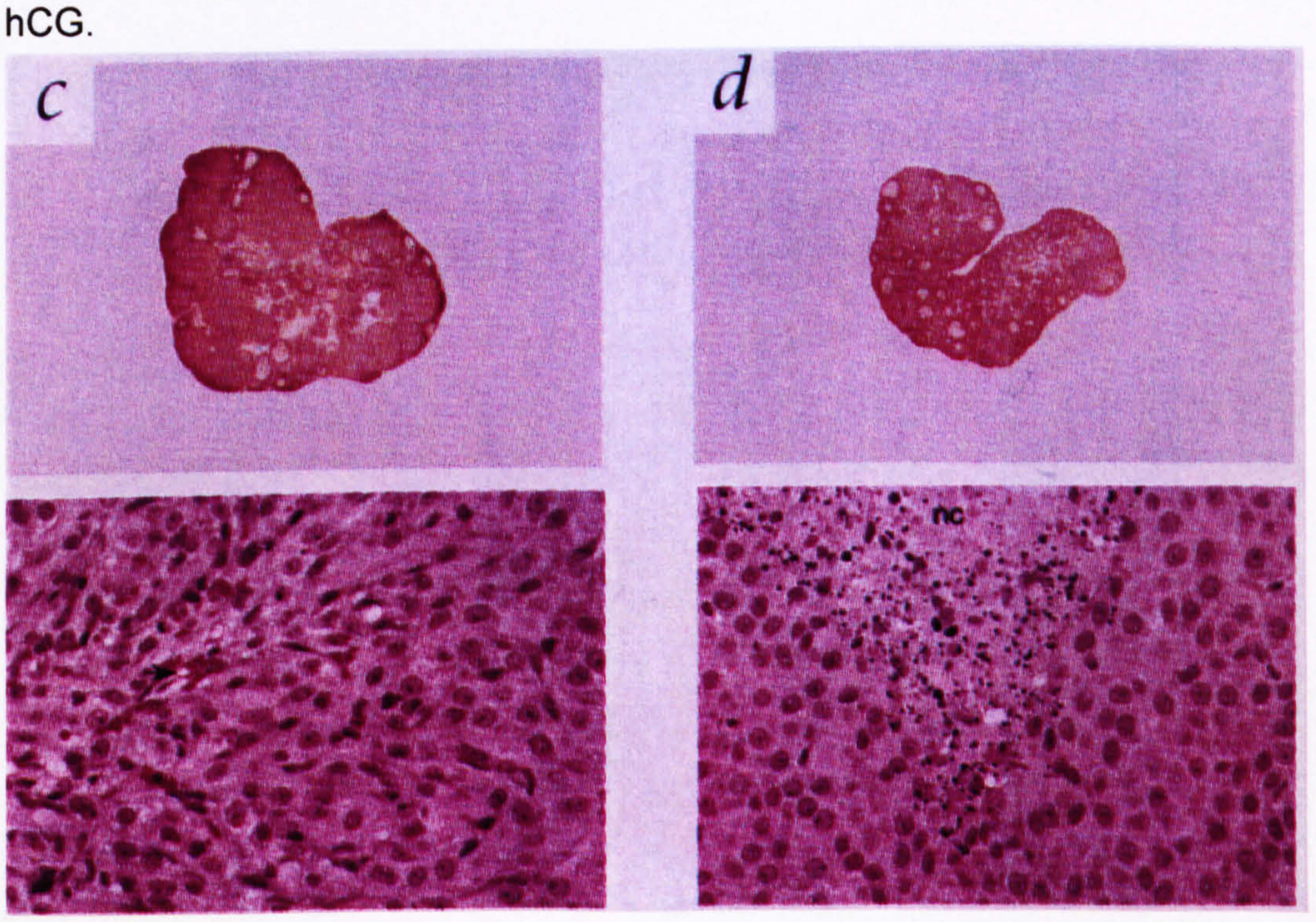


Figure 9.2.4(c,d)

Histological section of a murine ovary with the formation of corpus lutea. Both ovaries were superovulated with gonadotrophins and hCG but treated with c) human or d) murine Flt-IgG which are VEGF antagonists. Corpora lutea in both ovaries underwent regression and necrosis after treatment with VEGF antagonists.Necrosis was more pronounced after treatment with murine Flt-IgG. (Figures 9.2.4 a-d adapted from Ferrara *et al* 1998)

2.2 Angiogenesis within the endometrium:

The endometrium and the endometrial blood vessels undergo cyclical changes in their structure and secretory activity in correlation with the cyclical growth and maturation of ovarian follicles and are under the control of hormonal changes.

After menstruation endothelial cells proliferate from the ruptured arterioles and venules and recruit other cells, such as pericytes, for vessels to become capillaries and smooth muscle cells to become larger blood vessels. VEGF and its receptors Flt-1 (VEGF-R1) and KDR (VEGF-R2) along with angiopoetin 1 and 2 which bind to endothelial specific TKR, tie-2, interact to regulate blood vessel formation and degradation (Hanahan, 1997).

Lengthening and coiling of the spiral arteries within the endometrium is under the influence of oestrogen and progesterone, beginning during the proliferative phase of the menstrual cycle, continuing into the secretory phase. All transcripts of VEGF (121, 145, 165, 189 but not 206) were found to be expressed in the oestrogen responsive, proliferative columnar epithelium (i.e., luminal and glandular), lining both oviducts and the uterus (Charnock-Jones et al., 1993, Li et al., 1994, Shifren et al., 1996) and within the myometrium. The major transcripts expressed were $VEGF_{165}$ and $VEGF_{121}$. However during the secretory phase of the menstrual cycle, the site of VEGF expression shifts to cells of the underlying stroma, composing the functional endometrium (Charnock-Jones et al., 1993, Torry et al., 1996). VEGF in the stroma may serve as a source of angiogenic activity that supports extension of the stromal vessels. Therefore although VEGF immunoreactivity can be detected in the glandular epithelium throughout the menstrual cycle, cyclical variation is observed in the endometrial stromal tissue and blood vessels. This cycle dependent expression of VEGF in the endometrium suggests that VEGF is hormonally regulated.

Within the rat uterus an increase in VEGF mRNA was observed within 1-2 hours of the application of oestradiol and progesterone (Cullinan-Bove and Koos, 1993, Hyder et al., 1996). This response suggests that VEGF may

play a role in increased vascular permeability and proliferation of the uterine blood vessels. Oestrogen by itself is not mitogenic to endometrial cells (Cooke et al., 1986, Inaba et al., 1988) but regulates the synthesis of paracrine and autocrine growth factors by uterine cells which have mitogenic action. Hyder et al., (1996) also observed that the oestradiol increase in VEGF expression is inhibited by actinomycin - D but not by puromycin, suggesting that the effect is in part at least, due to direct oestrogen receptor regulation of VEGF transcription. Anti-oestrogens, tamoxifen, nafoxidene and clomiphene displayed a similar inhibition (Hyder et al., 1996). The antiangiogenic action of anti-oestrogens does not occur via oestrogen receptor but by a direct inhibition of growth factor stimulated endothelial cell growth (Gagliardi et al., 1996).

In the monkey, mifepristone (RU - 486) reduced endometrial glandular staining for VEGF indicating that anti-angiogenic effects of mifeprostone via suppression of VEGF production may represent a mechanism for its quelling effect on the endometrium (Greb et al., 1997). It is likely that VEGF mRNA expression within the endometrium is also regulated by non-hormonal events such as reduced oxygen tension, i.e., hypoxia, (Sharkey et al., 1998) induced at the time of menstruation and several other growth factors expressed in the human endometrium along with their cognate receptors. These include EGF, TGF-alpha, PDGF, FGF (Stavri et al., 1995), TGF-beta, (Petrovaara et al., 1994), IGF-I (Warren et al., 1996), IL-1, (Li et al., 1995), IL-6 (Cohen et al., 1996), IL-8 and PGE_2 (Laitinen et al., 1997).

Angiogenesis within the endometrium has not been addressed in this thesis. The possible contribution of the uterus to circulating VEGF, though, has been explored and is described later.

2.3 Angiogenesis in the embryonic implantation sites:

(refer to section on 'Role of VEGF in pregnancy', page. 74)

2.4 Expression of VEGF in other steroidogenic cells:

Other steroidogenic cell types such as the cortical cell of the adrenal glands

also express VEGF mRNA. Non steroid producing cells of the adrenal gland i.e., the medulla do not express VEGF mRNA.

2.5 Role of VEGF in supra-physiological and pathological angiogenesis in the female reproductive system:

The relevance of polycystic ovaries, in-vitro fertilisation, ovarian hyperstimulation syndrome, effect of hysterectomy and of menopause and hormone replacement therapy to circulating VEGF concentrations has been addressed, in studies constituting the thesis. These conditions are therefore briefly described here.

2.5.1 Polycystic ovaries (PCO):

In 1995, Kamat and colleagues observed that VEGF mRNA is overexpressed in the hyperthecotic stroma of 3 polycystic ovaries (Fig 9.2.5 b) but not in normal ovaries (Fig 9.2.5 a). They hypothesised that the dense vascular stroma, which is characteristic of PCO could result from this overexpression. Further Zaidi and colleagues in 1995 reported that women with PCO and PCOS had higher blood flow velocities in the ovarian stroma compared with women with normal ovaries (Fig 9.2.6).

The author has attempted to explore the causal factors of these important findings, in the research associated with the thesis. In the present part of the thesis the scientific context of the PCO is briefly described.

Polycystic ovary syndrome (PCOS) is a frequently encountered endocrinopathy in women of reproductive age and is the most common cause of anovulatory infertility. It is a heterogeneous condition with a broad spectrum of clinical and biochemical features (Jacobs, 1987, Franks 1995). Symptoms vary from slight menstrual disturbances, acne and/or hirsutism to the full syndrome characterised by Stein and Leventhal in 1935 as the presence of bilateral polycystic ovaries (PCO) associated with menstrual disturbances, anovulation and infertility, hirsutism, acne and obesity (Stein and Leventhal. 1935). Endocrinologically the syndrome is characterised by hyperandrogenism, inappropriate gonadotrophin secretion and hyperinsulinaemia. Biochemical markers of PCOS are elevated luteinising hormone and/or androgens and/or hyperinsulinaemia.

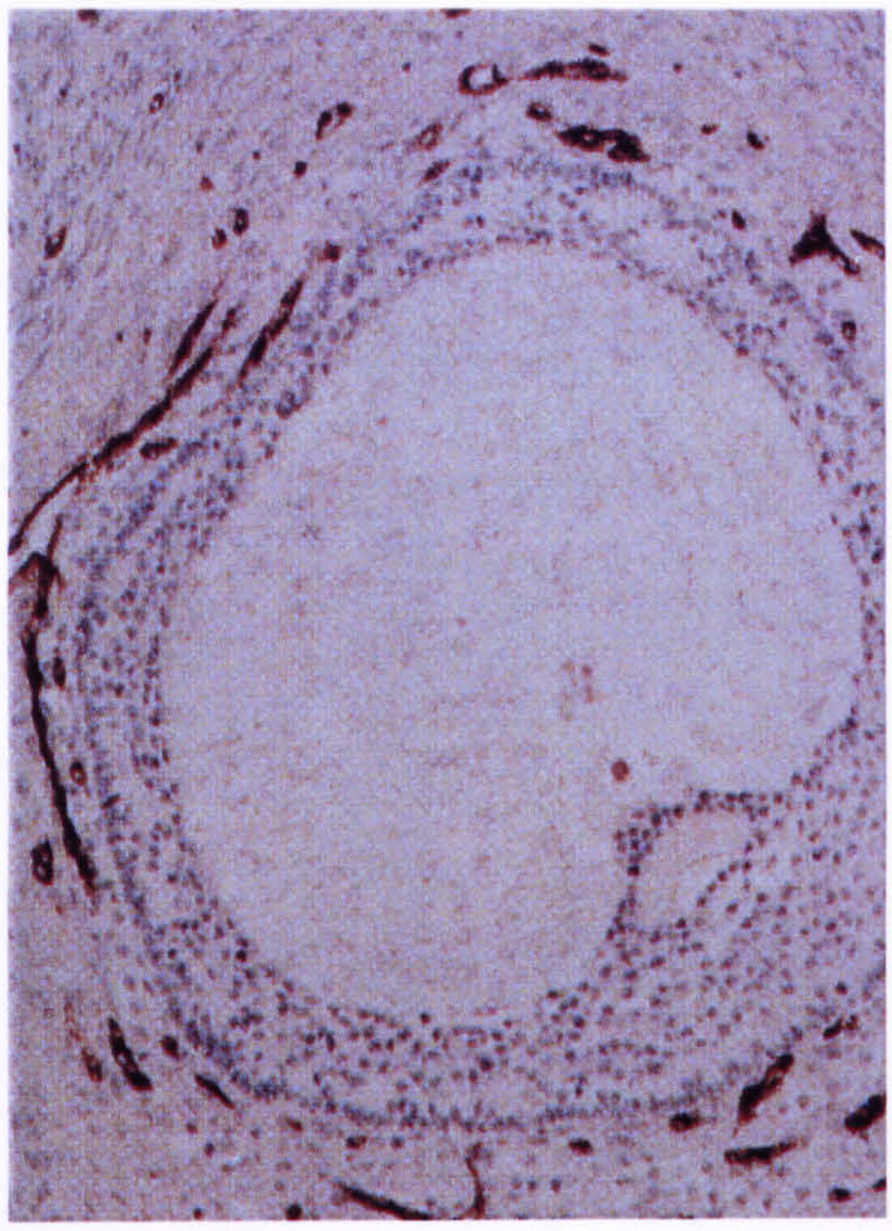


Figure 9.2.5(a)

Immunohistochemical staining with avidin-biotin perioxidase conjugate for VEGF in a healthy human ovary. (a) VEGF is confined to the blood vessels surrounding the preovulatory follicle in a normal ovary. Primary antibody used was an affinity purified rabbit antibody raised against VEGF. Magnification x155

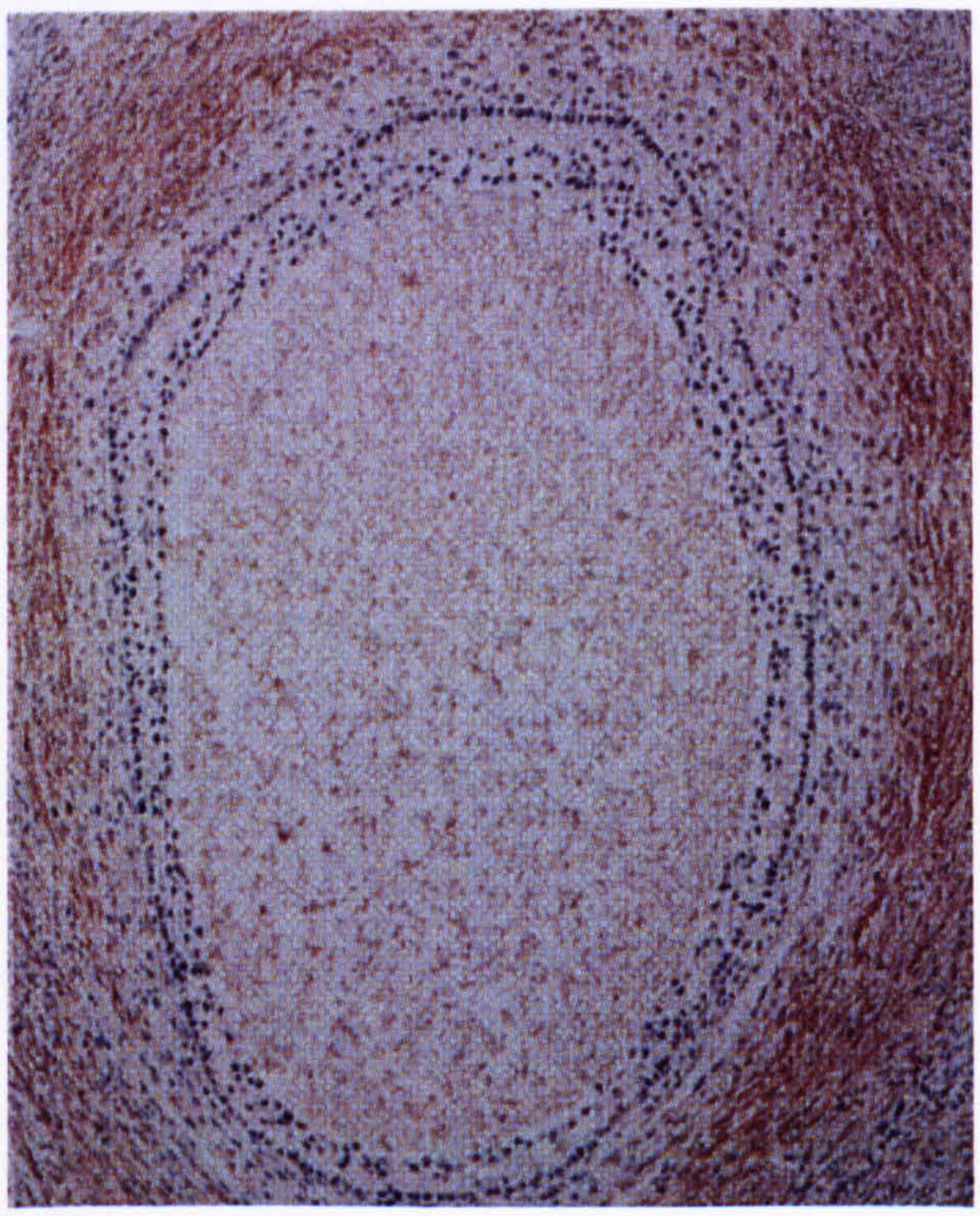


Figure 9.2.5(b)

Immunohistochemical staining for VEGF in a healthy human ovary. b) Intense staining for VEGF in the hyperthecotic stroma of a polycystic ovary. (Figures 9.2.5 a and b adapted from Kamat *et al* 1995)

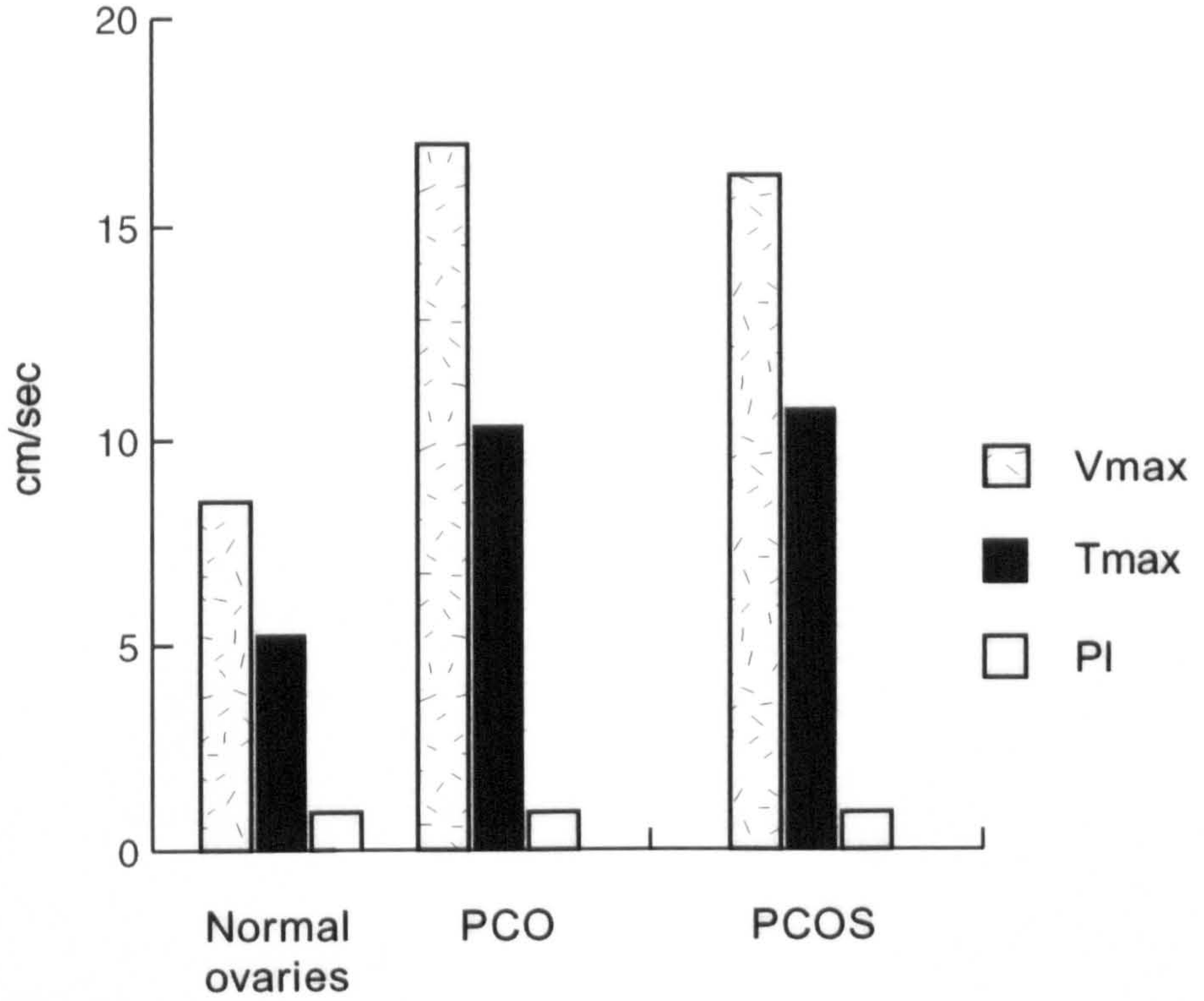


Figure 9.2.6

Doppler blood flow velocities and pulsatility index in the ovarian stroma of women with normal ovaries, PCO and PCOS.
(Zaidi *et al* 1995)

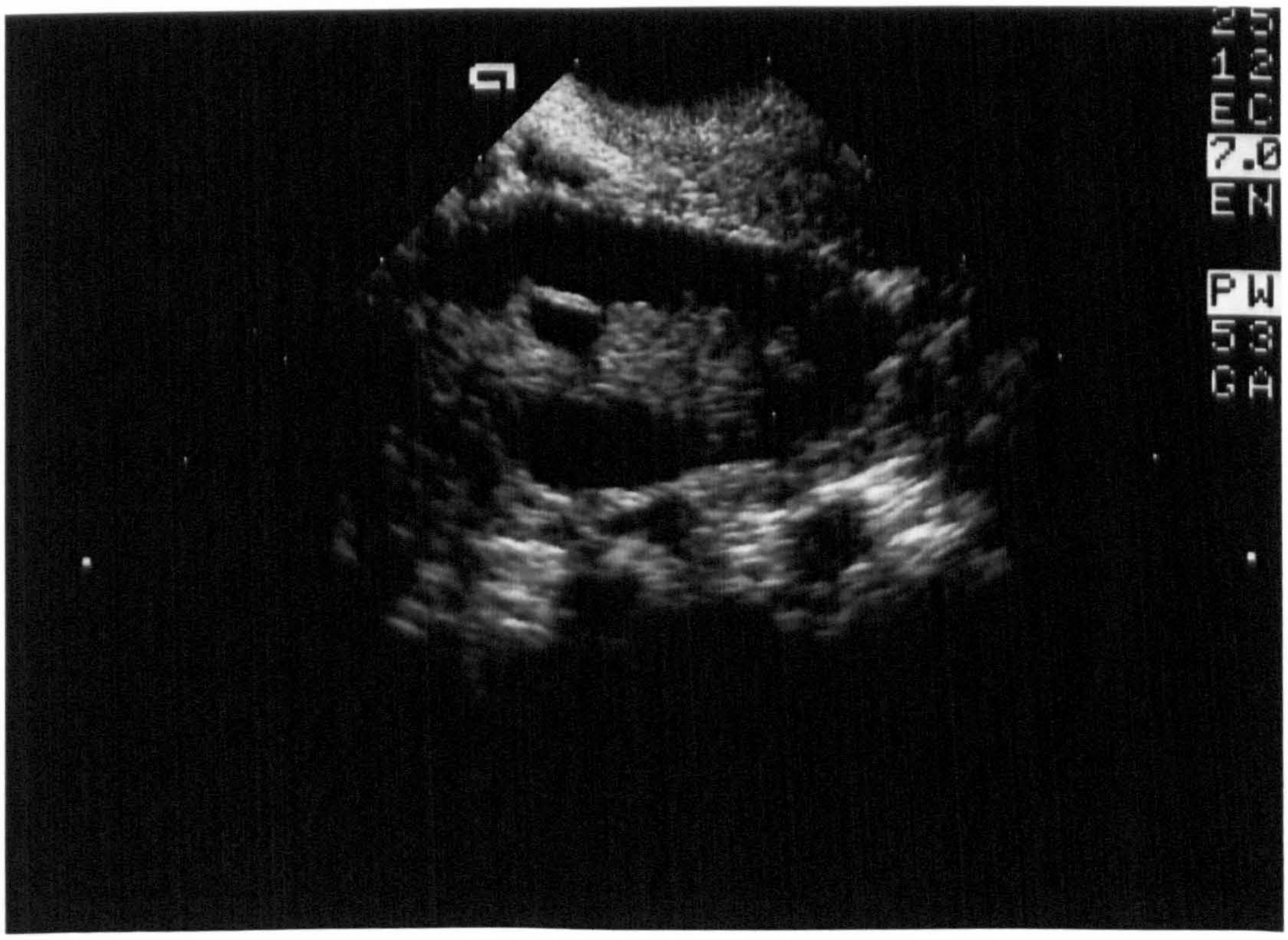


Figure 9.2.7

Ultrasound image of a polycystic ovary which may be described as an ovary with increased ovarian volume, increased density of ovarian stroma and more than 10 follicles as measured in one plane.

Clinical and biochemical heterogeneity of the condition has led to controversy in the definition of the disorder. The view taken in the author's department and followed in this thesis is that women are classified as having PCO if they have PCO morphology on ultrasonography and as having PCOS if they have PCO on ultrasound in addition to isolated or multiple clinical and/or biochemical markers of the syndrome.

Morphology of the PCO:

A classical polycystic ovary on histological examination, as described by Stein and Leventhal, 1935, was enlarged, with 20 -100 cysts per ovary which were located near the surface of the ovary, usually confined to the cortex. It had a thick fibrous tunica (50% increase) and stromal hyperplasia (33% increase). Other histological studies on PCO confirmed similar findings of enlarged ovaries, thickened and collagenised tunica, with increased cortical and subcortical stromal tissue which was at least 5 times greater than normal. The PCO had twice the number of follicles (primary, secondary and tertiary) but the same number of primordial follicles but not preovulatory follicles (Goldzieher and Green, 1961, Hughesden, 1982).

In early ultrasound studies of PCO, the main diagnostic criterion was ovarian size. The typical PCO was said to be 2-5 times larger than a normal ovary (Orsini al., 1985), a mean ovarian surface of 17 cm^2 (Parisi et al., 1982) and a mean ovarian volume of about 12 ml (Swanson et al., 1981). Later studies demonstrated that at least one third of patients with PCOS had ovaries of normal size (Orsini et al., 1985). More recently ultrasound assessment has focused on the increased number of cysts and stromal characteristics which have become the most important ultrasonic diagnostic criteria of PCO, particularly when the ovary is of normal size.

With the advent of high-resolution ultrasound scanning, a working definition of PCO based on ultrasound appearance has developed. It relies on the presence of 3 or more of the following criteria; enlarged ovarian volume (>9 ml), ten or more follicles of 2-8 mm in diameter in one plane, increased density and volume of stroma and thickened tunica as detected by trans-

abdominal or trans - vaginal ultrasonography (Adams et al., 1985, Fox et al., 1991) (Fig 9.2.7). Recently, Takahashi et al., 1994, compared histological findings of PCO and normal ovaries with ultrasonographic morphological features using transvaginal ultrasound and noted that the number, size and position of the small cysts on ultrasound correlated with histological findings. They concluded that ultrasound assessment of ovarian appearance was specific enough to allow a histological diagnosis of PCO without the need for ovarian biopsy. Borderline PCO may be defined as ovaries with thickened ovarian stroma and / or tunica but the number of follicles observed in one plane are between 6 and 10.

Prevalence:

By using pelvic ultrasonography it has become evident that PCO is a common finding in the general population, with up to 22% of volunteer women having a polycystic ovarian morphology (Polson et al., 1988). Some women had neither clinical or biochemical feature of PCOS while most had some symptoms or at least one biochemical marker of the syndrome. Though PCO occur in 22% of normal women, it has been observed in 40% of patients undergoing IVF, irrespective of the indication of treatment (MacDougall et al., 1993).

Endocrine profile:

The endocrine profile of women with PCOS is reported as having a varying degree of hyperandrogenism (22-78%), hypersecretion of LH (33%-66%) (Conway et al., 1989, Franks et al., 1989, Obhrai et al., 1990, Robinson et al., 1992), normal or low concentrations of FSH (Yen et al., 1970, Fauser et al., 1991) and hyperoestrogenaemia (Fox et al., 1991). These biochemical markers may appear in isolation or together.

<u>Elevated Androgens:</u>

Hyperandrogenism (raised serum free testosterone and/or androstenedione) is the most frequent finding in women with PCO (Franks, 1989, Robinson et al., 1992). It accounts for the clinical findings of hirsutism, acne and androgen dependent alopecia and is associated with a higher body mass

index (BMI), increased ovarian stromal volume and increased serum LH concentrations (Franks et al., 1989). Women with type A insulin resistance secondary to mutations in the insulin receptor gene may have serum testosterone concentrations in the male range and are also likely to have acanthosis nigricans (Conway and Jacobs, 1993).

Elevated LH:

Elevated LH has been observed in 25%, 33% and 45% of women with PCO (Polson et al., 1988a, Robinson et al., 1992, Conway, 1989). This endocrine feature may result in reduced conception rates and increased rates of miscarriage in both natural and assisted conception (Balen et al., 1993).

When the above two biochemical features are considered together, 86% of women with an ultrasound finding of PCO have either a raised testosterone, androstenedione, LH or a combination of these findings (Robinson et al., 1992).

Hyperoestrogenism:

Women with PCO are often referred to as being "hyperoestrogenised" (Fox et al., 1991) on the basis of a thickened uterine endometrium and increased serum oestrone concentrations. Oestradiol concentrations in anovulatory women with PCOS are typically in the range of early to mid-follicular phase levels of normal ovulatory cycles (Polson et al., 1987). Oestrone concentrations, in overweight patients particularly, however tend to be elevated due to the peripheral conversion of androstenedione to oestrone in adipose tissue (Baird et al., 1977, Yen, 1980. Polson et al., 1987). There may be no cyclical changes in oestrogens or increase in progesterone, therefore the problem in anovulatory women with PCO is often one of unopposed oestrogens (Yen, 1980).

Hyperinsulinaemia:

Insulin resistance with compensatory hypersecretion of insulin are features of PCOS (Burghen et al., 1980) and are associated with anovulation in PCOS (Dunaif et al., 1987, Conway et al., 1990, Sharp et al., 1991,

Robinson et al., 1993). Therefore the possibility exists that insulin is involved in the mechanism of anovulation. Hypersecretion of insulin in women with PCO, particularly in obese women, stimulates ovarian secretion of androgens, leading to hirsutism, menstrual disturbances, infertility and acanthosis nigricans (Conway and Jacobs, 1992). Increased BMI is correlated with an increased rate of hirsutism and serum testosterone concentration, cycle disturbance and infertility. Loss of weight in obese women (BMI > 30 kg/m2) often improves the endocrine profile (Kiddy et al., 1989). The prevalence of diabetes in obese women with PCOS in one survey was 11% (Conway et al., 1996) and so assessment of glucose tolerance is important in these women.

Aetiopathogenesis of PCO(S):

The aetiopathogenesis of PCO(S) remains unknown. Whether the primary cause of PCO/PCOS is a hypothalamic, adrenal or an ovarian defect is unclear. PCOS is a familial condition, which has led to the hypothesis that the polycystic ovary is a morphological manifestation of a genetically determined disorder of androgen biosynthesis and that the heterogeneous nature of PCOS is best explained by the interaction of this central disorder with environmental and other genetic factors. The observation of familial aggregation of PCOS (Hague et al., 1988, Legro et al., 1998) is consistent with a genetic basis of the disorder. However the mode of inheritance of PCOS has not yet been established. Although some studies support a single dominant gene with high penetrance (Legro et al., 1998, Carey et al., 1993), others do not (Jahanfar et al., 1995). It was proposed that the male phenotype may be premature male pattern baldness associated with elevated serum androgens (Ferriman and Purdie, 1979). Carey et al., 1994, identified a new single base change in the 5' promoter region of CYP17, the gene encoding for P450c 17 alpha on chromosome 10q24,3 in individuals with PCOS/male pattern baldness. While this variation in the A2 allele of CYP17 may cause modification of expression, it has been excluded as the primary genetic defect.

Several aetiopathological pathways for the development of PCOS have been

described. These include metabolic or regulatory pathways for steroid hormone synthesis (Gharani et al., 1997, Carey et al., 1994), for gonadotrophin action (Franks, 1995), the insulin- signalling pathway (Dunaif et al., 1992 and 1995) and pathways regulating body weight (Kiddy et al., 1992). Several genes from these pathways have been tested as candidate genes for PCOS (Gharani et al., 1997, Carey et al., 1994, Conway et al., 1994, Talbot et al., 1996, Sorbara et al., 1994, Taylor et al., 1992, Krook et al., 1994, O'Rahilly et al., 1991), in particular insulin receptor gene pathway. The possible causative genes and the mode of inheritance have recently been investigated by Urbanek et al., 1999. The authors carried out a genetic analysis of 37 candidate genes and tested for linkage with candidate genes by the affected sib pair test and for association between alleles of the candidate gene markers by the transmission / disequilibrium test. The 37 genes mapped to 33 distinct chromosomal locations. The evidence for linkage was observed with follistatin and CYP11A genes. However only linkage with follistatin remained significant after correction for multiple testing. Gharani and colleagues (1997) observed linkage with the CYP11A gene. Urbanek et al., 1999, however, observed no allelic association in the follistatin and CYP11A regions. They suggested that a combined analysis of linkage and association could provide evidence that one or several candidate genes contributed to susceptibility, even though the precise genetic variant is not known at the present time. Their results suggest that variation at or near the follistatin gene contributed to the hyperandrogenism of PCOS.

<u>Hypothalamic control:</u>

LH secretion has been used as an indicator of hypothalamic function in women with PCO. Although raised serum LH concentration is a characteristic of many women with PCO (Yen et al., 1970, Adams et al., 1986, Conway et al., 1989, Fauser et al., 1991), it is still disputed whether this reflects a primary or secondary hypothalamic-pituitary disorder. It is generally agreed that LH pulse amplitude is increased but the pulse frequency has been reported as both normal (Kazer et al., 1987) or increased (Waldstreicher et al., 1988) in women with PCOS. Some studies

have demonstrated an increased LH pulse amplitude *and* frequency, thus supporting a primary intrinsic hypothalamic-pituitary defect in PCOS. Although an increased LH response to GnRH in women with PCOS has been demonstrated (Rebar et al., 1976), abnormal LH secretion could be secondary to abnormal steroid feedback, such as unopposed oestrogens (Rebar et al., 1976) on the hypothalamo-pituitary axis rather than a primary disorder. After induction of an ovulatory cycle in women with PCOS, LH secretion and the exaggerated LH response to GnRH may be normalised (Blankstein et al., 1987). The above observations, which suggest that an abnormal gonadotrophin secretion is a consequence of the abnormal steroid environment in women with PCO, argue against a primary role of LH in the aetiology of PCO. Although women with PCO may have an elevated LH, a proportion of the women with PCO have normal basal and pulsatile LH secretion (Yen 1980, Adams et al., 1986, Conway et al., 1989). Furthermore, PCO have been observed on pelvic ultrasonography in women with hypogonadotrophic hypogonadism, despite little or no exposure to endogenous gonadotrophins in adult life (Shoham et al., 1992). Therefore it is highly unlikely that raised LH primarily causes PCO.

Androgens:

Hyperandrogenaemia is the most common endocrine finding in women with PCO (Franks, 1989). It has therefore been proposed that raised androgens may cause PCO. A higher prevalence of PCO in women with ovarian secreting tumours (Givens et al., 1974), in women with congenital adrenal hyperplasia and in female to male transsexuals (Spinder et al., 1989) provides circumstantial evidence for a role of androgens in the aetiology of PCO. The principal site of excess androgen production appears to be the ovary but there is also evidence in some patients for hypersecretion of adrenal androgens.

a. Adrenal androgens:

The evidence indicating an adrenal source of androgens may be related to the increased prevalence of PCO in women with congenital adrenal hyperplasia (CAH), elevated levels of dihydroepiandrosterone sulfate (DHEAS), which is an adrenal androgen and increased urinary excretion of

cortisol and adrenal metabolites in some women with PCO (Hoffmann et al., 1984, Rodin et al., 1994). In another study, however, no difference was observed between women with PCO and controls in the adrenal specific metabolite of androstenedione, 11-hydroxy-androstenedione (Polson et al., 1988 b). The studies on adrenal androgens remain inconclusive, since suppression of adrenal function with dexamethasone has been reported to both normalise (Loughlin et al., 1986, McKenna and Cunningham, 1992) and not normalise androgen levels in women with PCO (Lachelin et al., 1982). Similarly hyper-responsiveness of adrenal androgens to adrenocorticotrophic hormone (ACTH) has been reported in some but not all women with PCO (Loughlin et al., 1986, McKenna and Cunningham, 1992)

b. Ovarian androgens:

PCOS is predominantly a problem of ovarian hyperandrogenism, probably resulting from a primary abnormality of androgen biosynthesis. It may occur because of dysregulation of ovarian androgen production caused by a hyperactivity of the P450c17 alpha complex (Rosenfield et al., 1990). Cytochrome P450c17 alpha is the key enzyme involved in ovarian androgen biosynthesis in the ovary. It is expressed almost exclusively in the thecal cell layer of the developing follicle and is LH dependent. It is responsible for 17-hydroxysteroid dehydrogenase and 17, 20 lyase whose putative increased activity in the polycystic ovary induces an overproduction of 17-hydroxyprogesterone and androstenedione. Gilling-Smith et al., (1994), using monolayer cultures of thecal cells in a serum-free medium, have demonstrated hypersecretion of androstenedione per thecal cell from polycystic ovaries compared with normal ovaries, consistent with the theory that there is a primary abnormality in the regulation of androgen production in PCOS. This proposed dysregulation of P450c17 alpha activity could be influenced by insulin and/or biologically active IGF's in combination with LH. Polymorphism of the gene for this dysregulation however, shows no association with PCOS.

<u>VEGF:</u>

It was demonstrated that VEGF mRNA is overexpressed in the hyperthecotic ovarian stroma of 3 women with polycystic ovaries (Kamat et al., 1995).

Gordon et al., 1996, suggested that the increased expression of VEGF within the stroma of PCO could be stimulated by LH. LH is also known to promote angiogenesis and modulate the expression of VEGF within the ovary (Ravindranath et al., 1992, Dissen et al., 1994,).

In the in-vitro experiments of the thesis, investigation of insulin and testosterone action on VEGF secretion by luteinised granulosa cells has been described. The role of insulin and androgens in PCO(S) is therefore briefly described.

Androgens:

Plasma testosterone tends to be higher in anovulatory than ovulatory women with PCO (Franks, 1991) but excess androgens are unlikely to be the underlying cause of anovulation. Weight matched ovulatory and anovulatory women with PCO have been found to be equally hyperandrogenic (Conway et al., 1989, Sharp et al., 1991, Robinson et al., 1993). It has also been shown that theca cells from PCO when cultured in-vitro, hypersecrete androgens compared with theca cells from normal ovaries (Erickson and Yen, 1984, Gilling-Smith et al., 1994) regardless of the ovulatory status of the women from whom the ovaries were obtained (Gilling-Smith et al., 1994). This suggests that hyperandrogenism in PCO is an intrinsic defect associated with the ovarian morphology and is not a function of the ovulatory status of the patient.

Insulin:

PCO are common in insulin resistant women with acanthosis nigricans (Kahn et al., 1976) and hyperinsulinaemia and insulin resistance are features of PCOS (Burghen et al., 1980). In recent studies it has become apparent that hyperinsulinaemia and insulin resistance are associated with anovulation in PCOS (Dunaif et al., 1987, Conway et al., 1990, Sharp et al., 1991, Robinson et al., 1993). Insulin may be involved in the mechanism of anovulation in PCOS. A post - receptor defect unique to PCOS, causing resistance to insulin action has been described. Dunaif et al., 1992 have described an abnormality of insulin receptor phosphorylation, suggesting

down-stream defects in the insulin-signaling pathway. The resulting compensatory hyperinsulinaemia occurs in the majority of obese women with PCOS but is also a common finding in lean women who have the syndrome. In PCOS there is a marked depression of circulating IGF-binding protein-1 (IGFBP-1) concentrations. These are inversely correlated with insulin concentrations and these largely interrelated disorders may play a significant role in the pathophysiology of PCOS. Insulin inhibits IGFBP-1 gene expression, and these changes may reflect insulin sensitivity at the hepatic level, despite insulin resistance in other tissues in the majority of PCOS patients. Aberrant regulation of IGFBP-1 may play a role in the pathogenesis of this disorder. Concurrently with decreased IGFBP-1 are elevated levels of free IGF-1, which may act synergistically with LH to stimulate thecal androgen production.

2.5.2 Ovarian stimulation for in-vitro fertilisation (IVF) and the ovarian hyperstimulation syndrome (OHSS):

Production and secretion of VEGF by luteinised granulosa cells occur after ovulation and are regulated by LH or hCG. The findings of the first clinical study in the thesis on changes in serum VEGF concentrations during the normal menstrual cycle support this observation.

It was therefore hypothesised that with an increase in the mass of granulosa cells, as seen in regimens of ovarian stimulation used for in -vitro fertilisation (IVF) where women produce multiple follicles and therefore multiple corpora lutea, production of VEGF may be enhanced. In two of the clinical studies reported in this thesis, serum VEGF changes during IVF treatment using the "long protocol" of pituitary desensitisation using GnRH analogues, have been studied. The treatment protocol for IVF is described later.

It has been suggested that luteinising hormone (LH) may play a role in inducing hyperaemia within the CL (Niswender et al., 1976). To support this hypothesis, Ravindranath and colleagues (1992) demonstrated that treatment with GnRH antagonists resulted in a decline of VEGF mRNA within the primate ovary. They suggested that this fall of VEGF is more likely to be due to a suppression of pituitary gonadotrophin secretion rather than

the direct effects of the antagonists on the CL itself. We therefore hypothesised that treatment with GnRH analogues during pituitary suppression may result in fall of VEGF concentrations by a similar mechanism. The effect of pituitary suppression using GnRH analogues on serum VEGF concentrations is explored later in the thesis.

Ovarian stimulation during IVF treatment is achieved using gonadotrophins containing FSH and LH (human menopausal gonadotrophin, HMG). hCG is administered, 36 hours prior to scheduled oocyte retrieval, once adequate follicular growth is achieved. hCG causes final maturation of the oocytes. The effect of follicular growth on serum VEGF concentrations during treatment with gonadotrophins and hCG has also been explored. It was hypothesised that with the production of multiple follicles and therefore multiple corpora lutea and /or intensified sensitivity towards LH/hCG, as seen in women with PCO, larger amounts of VEGF would be produced. This may be responsible for the fluid shift from the vascular bed to the extravascular space, which characterises the syndrome of OHSS.

In women who developed OHSS, serum and follicular fluid VEGF concentrations and the release of VEGF by granulosa lutein cells in-vitro, have therefore been explored in the thesis.

OHSS is one of the most serious iatrogenic complications of ovulation induction. Its pathophysiology is unknown, treatment is nonspecific and the optimum measures lie in prevention (Schenker, 1993, Brinsden et al., 1995).

Classification:

Based on the severity of the signs and symptoms and the biochemical findings this syndrome has been traditionally graded into the mild, moderate and the severe categories (Golan et al., 1989).

Pathology:

Besides enormous cystic ovarian enlargement (greater than 5 cms in size) there is massive extravascular fluid shift and secondary intravascular fluid volume depletion (Elchalal and Schenker, 1997). This fluid is an exudate,

which accumulates primarily in the peritoneal, pleural and in the pericardial cavities causing a protein rich ascites, pleural and pericardial effusion (Fig 9.2.8 a, b, c, d). Loss of protein into the third space causes a fall in plasma oncotic pressure, which results in profound loss of intravascular fluid. Haemoconcentration results, which poses a risk of thromboembolism and suppression of urine formation causing renal failure (Elchalal and Schenker 1997, Rizk and Smitz, 1992).

Prevalence:

Most methods of ovulation induction can cause OHSS and the mild form may even result from the use of oral antioestrogen. In ovulation induction the risk is related to the dose of gonadotrophins and to the duration of treatment. The overall risk is estimated to be about 4% and that of severe form about 0.25 - 0.9%. In IVF the prevalence varies in published series from 6.6 - 8.4%, being the highest in those treatment protocols combining gonadotrophin with GnRH analogues. Severe cases occur in 0.5% - 4% of IVF cycles (MacDougall et al., 1993).

Risk factors:

a: Amongst the risk factors an important one is the presence of polycystic ovaries, which are known to make a multifollicular response rather than a unifollicular response (MacDougall et al., 1993) .

b: The development of a large number of small and intermediate follicles in response to gonadotrophin stimulation is an important risk factor. It is commonly seen in women with PCO (Fig 9.2.9) or too high a dose of gonadotrophins in women with normal ovaries (Navot, 1988).

c: Exposure of the ovaries to LH or hCG mediates the process of neovascularisation and vascular permeability. If the ovaries are not exposed to these agents OHSS does not occur except on rare occasions (Di Carlo et al., 1997). OHSS is therefore almost always preventable by withholding the hCG injection (Navot, 1988).

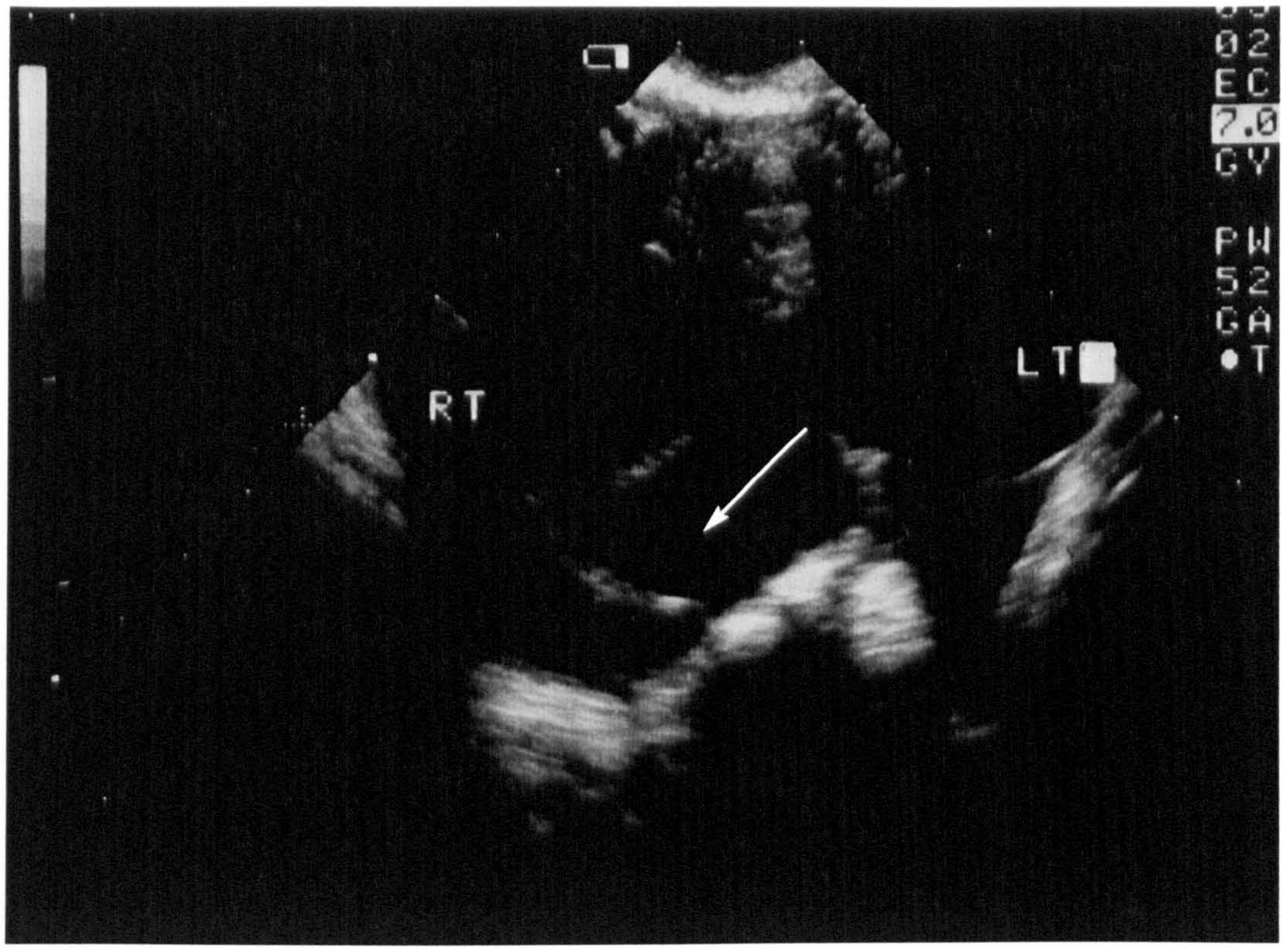


Figure 9.2.8(a)

Ovarian hyperstimulation syndrome. (a) Bilaterally enlarged cystic ovaries containing multiple luteal cysts.

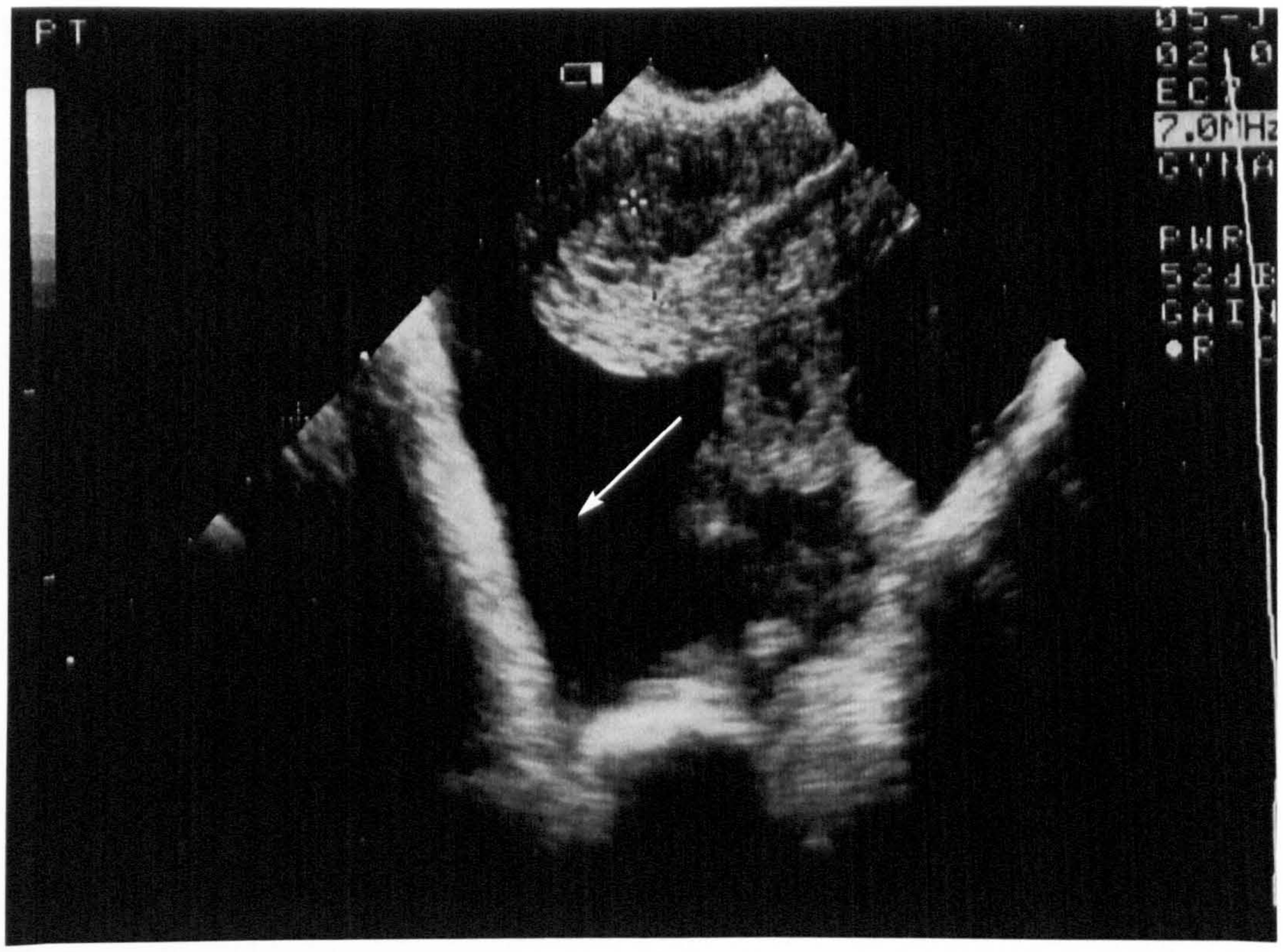


Figure 9.2.8(b)

Ovarian hyperstimulation syndrome. (b) Collection of fluid is seen behind the uterus in the Pouch of Douglas.

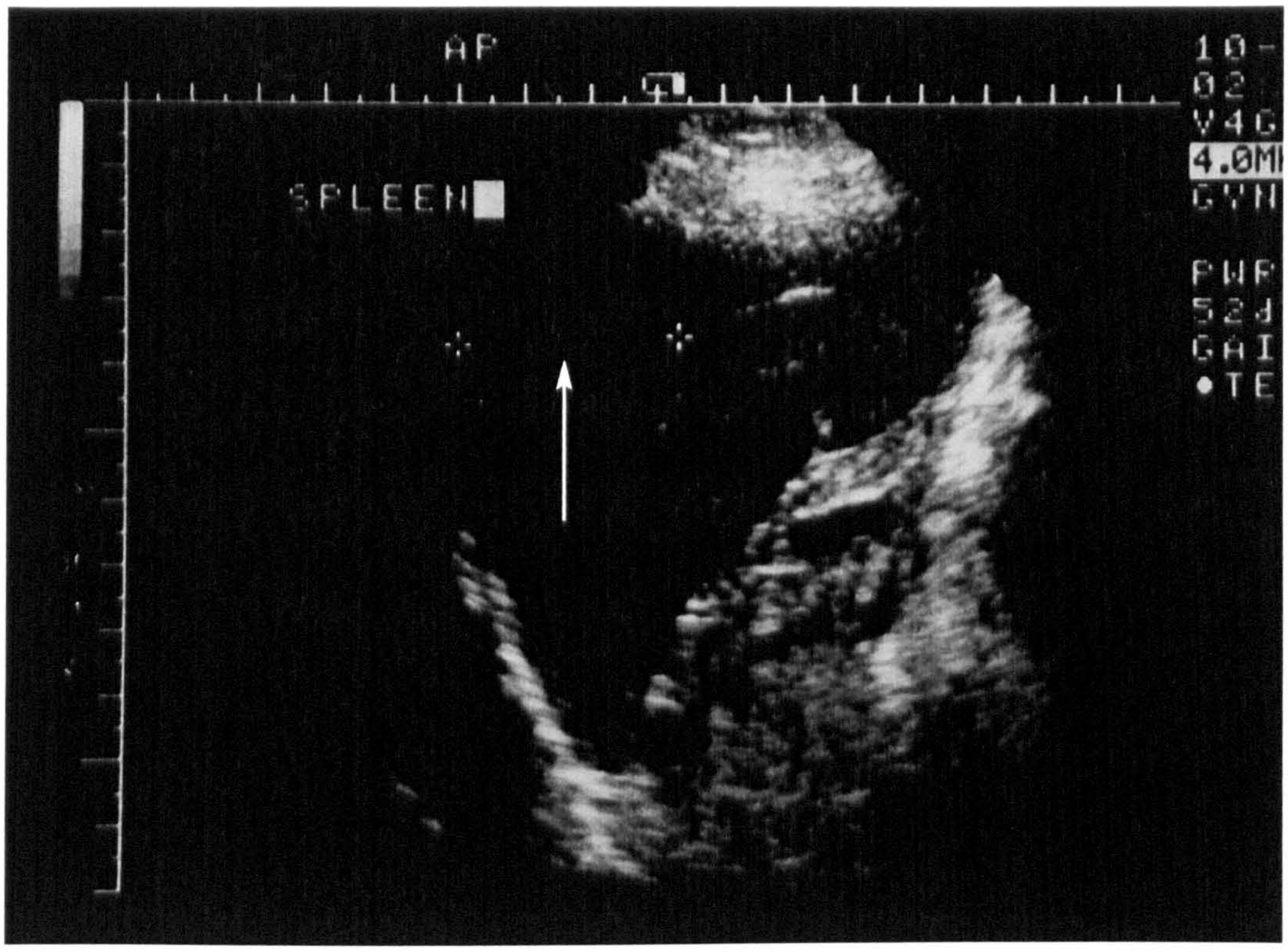


Figure 9.2.8(c)

Ovarian hyperstimulation syndrome. c) Ascites - collection of fluid in the abdomen below the spleen (marked between the 2 crosses).

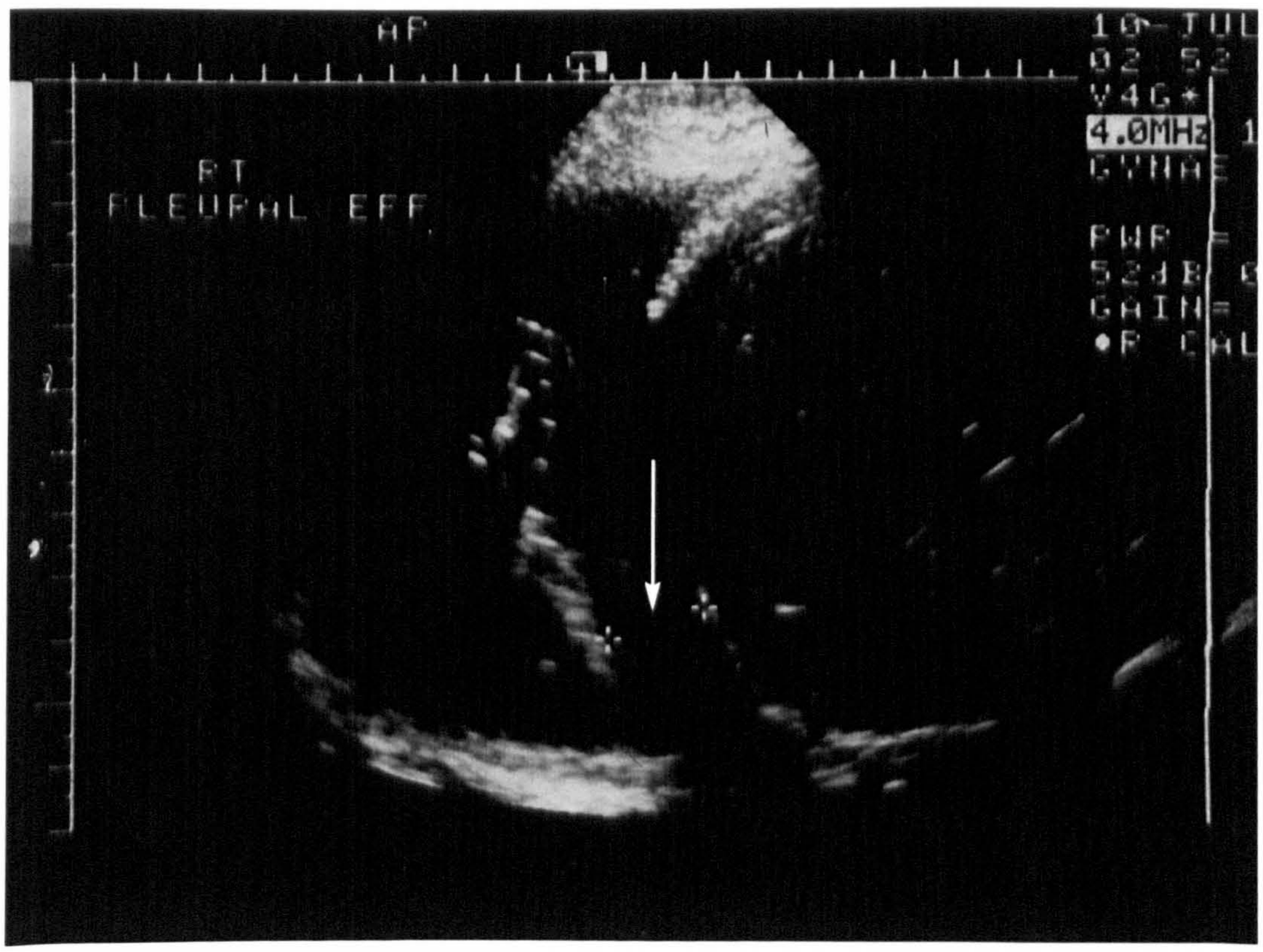


Figure 9.2.8(d)

Ovarian hyperstimulation syndrome. (d) Pleural effusion - collection of fluid between the lungs and pluera (marked between the 2 crosses).

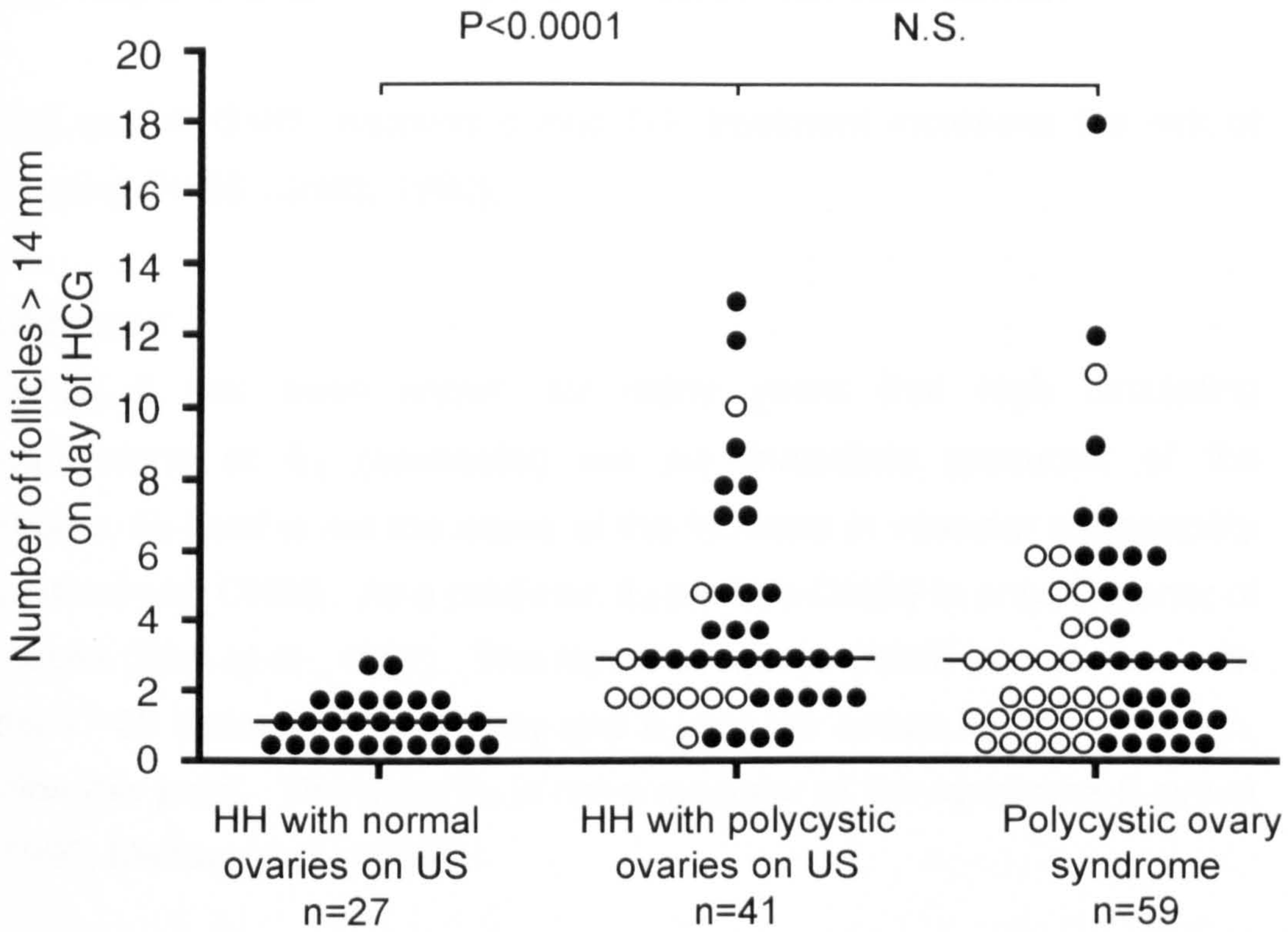


Figure 9.2.9

Multifollicular response in women with PCO and PCOS with hypogonadotrophic hypogonadism. Each dot represents the number of follicles greater than or equal to 14mm in diameter that developed after ovulation induction with HMG using either conventional (closed circles) or low dose (open circles) regimens. HH - hypogonadotrophic hypogonadism (Shoham *et al* 1992).

d: Most cases occur in younger and thinner women consistent with a greater ovarian responsiveness in this group compared with older women.

e: The use of GnRH agonists during IVF treatment increases the risk of developing OHSS (Smitz, 1990).

Mechanisms:

a: While it has been known for many years that high circulating concentrations of E_2 (oestradiol) are an immediate precursor of the syndrome, E_2 itself is not the cause of the increase in vascular permeability associated with OHSS. As a predictor, E_2 predicts OHSS in only a quarter of the cases (Rizk et al., 1997). The report of severe OHSS in a woman with partial 17-20 desmolase deficiency and a very low serum E_2 concentration, clarifies this point. Therefore E_2 is not a mediator of the syndrome (Levy et al., 1996, Meirow et al., 1996).

b: The ovarian renin angiotensin system was thought to be a causative factor. Excessive levels of ovarian derived renin and aldosterone were reported in the peritoneal fluid of a woman with severe ovarian hyperstimulation (Ong et al., 1991). Subsequent studies demonstrated that ascitic fluid in this syndrome contained large amounts of angiotensin II compared with ascitic fluid obtained from women with liver failure, thereby establishing a positive correlation between ovarian renin and severe OHSS (Navot et al., 1987).

c: Cytokines play an important role in ovarian paracrine interactions. The cytokines implicated in vascular permeability are TNF alpha, IL 1, IL 2, IL6 and IL8. Each of these cytokines has been shown to cause changes in vascular permeability but their combined effects may be additive (Loret-de-Mola et al., 1996). Endothelium derived relaxing factor, identified as Nitric oxide (NO), is also a recently recognised messenger molecule mediating vasodilatation and cytokine activities. It is postulated that NO and NO related metabolites may be involved in VEGF gene expression. Rizk and colleagues (1997) also confirmed the role of cytokines as a mediator of

OHSS.

Therefore any theory of pathogenesis of OHSS needs to accommodate three essential clinical observations. The first is that OHSS only occurs after ovulation or after administration of hCG, secondly that it is very much more common in women with PCO than in women with normal ovaries and thirdly that OHSS is a state of increased vascular leakage. The link between OHSS and VEGF may be provided by the fact that VEGF is an extremely potent promoter of angiogenesis and also renders endothelial cells hyperpermeable. The next link is provided by an important observation made recently by our group and others of increased ovarian stromal blood flow in women with polycystic ovaries.

The impact of hCG on granulosa cells can persist until the corpora lutea become insensitive to hCG, after 9 weeks of gestation. Clinical studies have shown that OHSS symptoms gradually diminish after the first trimester of pregnancy (Dahl-Lyons et al., 1994, Benifila et al., 1994).

In all of the studies in this thesis conducted on women, women with the following conditions were excluded from the studies because there is evidence to suggest that VEGF may be over-expressed in these conditions.

2.5.3 Benign ovarian cysts:

The manner in which fluid accumulates in benign cystic ovarian neoplasms is not well understood. Gordon et al., (1996), suggested that the ability of VEGF to increase permeability could lead to the formation of transudate in ovarian cysts and the Fallopian tube.

2.5.4 Endometriosis:

Active endometriosis is characterised by hypervascularisation of the implant and surrounding tissue. It was observed that peritoneal fluid from women with endometriosis had higher VEGF concentrations compared with women without the disease (Shifren et al., 1996). Cyclical fluctuations of VEGF concentrations in the peritoneal fluid were also observed in women with

endometriosis. These were not present in women without the disease. It was also suggested that the source of this VEGF could either be the implants or the peritoneal macrophages which are known to express VEGF (Shifren et al., 1996) and that the VEGF in peritoneal fluid could be the critical factor in the pathogenesis of endometriosis (McLaren et al., 1996).

2.5.5 Menorrhagia:

Pilot studies have shown that immunoreactivity for VEGF within the uterus is increased in women with menorrhagia (Smith, 1998).

2.5.6 Leiomyomata:

VEGF is expressed within leiomyomas to the same extent as the myometrium. There were no differences in the expression of VEGF mRNA during the various phases of the menstrual cycle (Harrison-Woolrych et al., 1994). Leiomyomata from women treated with a GnRH analogue did not have significantly different levels of VEGF mRNA from untreated leiomyomata (Harrison-Woolrych et al., 1994). VEGF however, may play a role in its pathogenesis, since the growth of these tumours is dependent on angiogenesis and is regulated by ovarian steroids.

2.5.7 Ovarian and uterine malignancies:

Within ovarian neoplasms, Flt-1 and KDR signals (VEGF receptor genes) were localised exclusively to endothelial cell (Abu-Jawdeh et al., 1996). Boocock et al., 1995, however found only KDR receptors to be located within vessel endothelial and tumour cells in primary and metastatic ovarian carcinoma. No receptors were located within benign ovarian neoplasms. Substantially higher concentrations of VEGF were detected in cyst fluids of malignant and borderline ovarian carcinomas than in fluid of benign cysts. There is evidence to suggest that VEGF may interact with other angiogenic factors in regulation of angiogenesis within ovarian neoplasms. These include PDGF (Reynolds et al., 1994, Anan et al., 1995), bFGF, EGF (Detmar et al., 1994), TGF- alpha (Detmar et al., 1994) and TGF- beta (Petrovaara et al., 1994). It is possible that these growth factors act directly or indirectly in upregulation of VEGF expression within tumour cells.

Hormonal regulation of VEGF expression is not yet proven. Kim et al., 1996, demonstrated that hormones such as oestradiol and progesterone did not regulate VEGF expression within endometrial cancers. The results of this study are in contrast to those of Charnock-Jones et al., 1993, who demonstrated that oestradiol increased steady state levels of mRNA encoding VEGF in a dose and time dependent manner within human endometrial carcinoma cell lines (HEC 1-A). Hyder et al., 1998, demonstrated progestogen regulation of VEGF expression within human breast cancer cell lines.

2.6 Role of VEGF in pregnancy:

Since there are data to suggest that VEGF may play a role in pregnancy, the presence of circulating VEGF in early pregnancy was explored. The role of VEGF in pregnancy associated problems was not studied in this thesis.

2.6.1 Angiogenesis in the embryonic implantation sites and placenta:

Under the influence of oestrogens and progestogens and after implantation of the blastocyst, endometrial stromal cells undergo transformation into decidual cells. Development of the decidua involves extensive proliferation of blood vessels associated with concurrent upregulation of VEGF expression in decidual cells (Jakeman et al., 1993, Shweiki et al., 1993, Chakraborty et al., 1995). VEGF secreted within the glandular lumina may potentially influence the nutrition and apposition of the developing blastocyst (Hornung et al., 1998). It was also suggested that the rapid growth of the placenta throughout gestation may be related to hypoxia induced angiogenesis mediated via VEGF (Wheeler et al., 1995).

In the human placenta, Clark et al., 1996 demonstrated that VEGF receptor Flt-1 mRNA was distributed around villous trophoblast (cytotrophoblast) mainly in the first trimester of pregnancy. Extravillous trophoblast (syncitiotrophoblast) expressed Flt-1 mRNA throughout gestation. In contrast, KDR mRNA was located only within endothelial cells. VEGF was also located within macrophages of both maternal and fetal macrophages in the decidua. PlGF and PDECF also coexpressed with VEGF. These growth factors play an important role in placental development and differentiation.

2.6.2 VEGF in embryonic development:

VEGF also plays a role in embryonic development. VEGF mRNA was found to be expressed in several organs of the mouse and rat embryo (Jakeman et al., 1993, Breier et al., 1992). In the human fetus (16 -22 weeks), VEGF mRNA expression is detectable in virtually all tissues, including the liver, kidney and spleen (Shifren et al., 1994).

Immunohistochemical analysis has shown the presence of VEGF protein

principally in embryonic epithelial cells, myocytes and smooth muscle cells lining the blood vessels but not in vascular endothelial cells (Shifren et al., 1994). These findings support the paracrine mechanism of action of VEGF whereby it is secreted by nonendothelial cells and modulates the activities of adjacent vascular endothelium.

There is evidence to show that Flk-1/KDR receptor expression is predominant in fetal, rather than adult tissues, thus suggesting an important role of Flk-1 receptors in fetal development which may be one of the earliest markers of endothelial cell precursors (Yamaguchi et al., 1993). VEGF receptor, Flt-1, on the other hand is selectively expressed in quiescent and proliferating endothelial cells and perhaps plays a role in maintenance of endothelial cells (Peters et al., 1993).

2.6.3 VEGF gene and receptor knockouts:

Both Flk-1/KDR and Flt-1 are essential for normal development of embryonic vasculature. Mouse embryos homozygous for a targeted mutation in the Flt-1 locus died between 8.5 to 9 days (Alon et al., 1995). Although endothelial cells developed in both embryonic and extra-embryonic sites they failed to organise in normal vascular channels (Fong et al., 1995). Mice in which the Flk-1 gene had been inactivated failed to develop blood islands. Haemopoeitic precursors were disrupted and vascular channels failed to develop in embryo or yolk sac resulting in death within 8.5 to 9 days (Shalabi et al., 1995). However, these findings do not necessarily imply VEGF as being essential since Flt-1 and Flk-1 receptors may be activated by other growth factors. However, recent studies have shown a direct role of the VEGF gene in embryonic development and vasculogenesis. Inactivation of the VEGF gene in mice resulted in embryonic lethality in heterozygous embryos between days 11 and 12. The VEGF +/- embryos were growth retarded and had a number of developmental anomalies (Theilier, 1989). These embryo however, survived for two more days than Flt-1 or Flk-1 null embryos. Therefore loss of even a single allele of the VEGF gene is lethal (Carmeliet et al., 1996, Ferrara et al., 1996)

2.6.4 VEGF in pregnancy associated problems:

VEGF-Flt-1 mediate calcium dependent nitric oxide release, which plays a role in trophoblastic invasion in normal pregnancy. Banks et al., 1998 found VEGF mRNA to be reduced in women with pre-eclampsia (Banks et al., 1998). They therefore suggested that trophoblastic invasion is defective in women with pre-eclampsia (Banks et al., 1998). In another study by Baker et al., 1995, serum VEGF concentrations were found to be elevated in women with pre-eclampsia compared with pregnant women without pre-eclampsia. Further studies are required to establish the role of VEGF in pregnancy related problems.

Section 3: Role of VEGF in the male reproductive system:

Within the testes, VEGF mRNA expression is confined to the specialised interstitial cells (Leydig cells) which produce the male hormone testosterone. No VEGF mRNA expression was observed within the seminiferous tubules (Shweiki et al., 1993).

Immunohistochemistry and in situ hybridisation studies on the prostate gland and seminal vesicles have demonstrated a strong expression of VEGF mRNA within these tissues. It has been suggested that semen concentration of VEGF contributes to an effect on male infertility. (Brown et al., 1996). The source of this seminal VEGF is thought to be the prostate gland and seminal vesicles.

Circulating serum VEGF concentration in men was explored and compared with serum VEGF concentrations in women in this thesis.

Experimental studies performed on rats have demonstrated that castration induced regression in the vasculature within the prostate gland, which was reversed by administrating testosterone. This study provides further evidence that testosterone may induce angiogenic factors such as VEGF within the male reproductive system (Franck-Lissbrant et al., 1998). There are no studies, however, to demonstrate that testosterone induces ovarian VEGF expression.

Section 4: Role of VEGF in other normal adult tissue:

Besides the reproductive system, VEGF mRNA is also expressed in lung, kidney, heart and adrenal glands and to a lesser extent in liver, spleen, gastric mucosa and breast (Berse et al., 1992). Using ISH, VEGF expression has been demonstrated in specific cell types, such as the cardiac myocytes, adrenal cortical cells, alveolar wall of the lung and in renal glomeruli. Microscopic location of these cells suggested they were epithelial in origin. Dermal papilla cells (DPC) also expressed functional VEGF receptors. VEGF in DPC serves as an autocrine growth factor promoting cyclical hair growth (Lachgar et al., 1996). VEGF is also expressed in osteoblasts. PGE_2 induced its expression in osteoblasts and glucocorticoids reduced it. This has led to a suggestion that VEGF may be involved in normal bone physiology (Harada et al., 1994). The role of VEGF within normal adult tissue is not yet known but it is possible that it serves to maintain the existing density of endothelial cells in normal tissue and may be responsible for inducing and maintaining baseline permeability of the normal circulation (Senger et al., 1993). VEGF has also been detected in cytoplasm of certain cells. It is possible that these could represent storage forms of VEGF, available for prompt secretion following local injury or malignant stimuli (Senger et al., 1993).

Section 5: Role of VEGF in pathological angiogenesis:

Pathological angiogenesis is characterised by persistent proliferation of endothelial cells.

A. Tumour angiogenesis:

Tumour growth is dependent of the vascular supply from the surrounding tissues to proliferate and metastasize. Tumour vasculature is characterised by increased permeability relative to normal vessels (Roberts and Palade 1997) which requires a constitutive activation of endothelial cells.

Although numerous angiogenic factors are involved, almost all tumours secrete VEGF. In situ hybridisation and immunohistochemical studies have demonstrated that VEGF mRNA expression is markedly upregulated in the majority of tumours. These include intracranial tumours, such as, glioblastoma multiforme, meningioma, capillary haemangioma (Plate et al., 1992, Berkman et al., 1993, Phillips et al., 1993), thyroid (Viglietto et al., 1995), lung (Mattern et al., 1996), breast (Brown et al., 1995) carcinomas, gastrointestinal tract and (Brown et al., 1993, Suzuki et al., 1996), urinary tract tumours (Brown et al., 1993), angiosarcoma (Hashimoto et al., 1995) and female reproductive tract tumours such as ovarian (Boocock et al., 1995), uterine endometrial (Charnock-Jones 1993, Guidi et al., 1995, Kim et al., 1996), cervical carcinomas (Olson et al., 1994), choriocarcinomas (Charnock-Jones et al., 1994) and germ cell tumours (Viglietto et al., 1996).

A correlation has been observed between tumour vascularity and VEGF mRNA expression (Suzuki et al., 1996, Guidi et al., 1995, Berkman et al., 1993). In tumours where VEGF and PlGF are coexpressed, only VEGF expression correlated with malignancy and vascularity (Viglietto et al., 1995 and 1996).

In tumour sections VEGF mRNA was expressed in tumour cells but not in endothelial cells. VEGF receptors, Flt-1 and KDR mRNA however, were expressed on endothelial cells (Brown et al., 1993, Plate et al., 1994). These findings support the role of VEGF as a paracrine mediator of angiogenesis (Ferrara et al., 1993). In an angiosarcoma (tumours of

endothelial cell origin) however, VEGF Flt-1 mRNA was expressed in the cells itself, suggesting an autocrine role of VEGF in mediating angiogenesis (Hashimoto et al., 1995). It has also been suggested that lymphocytes (Freeman et al., 1995) infiltrating the tumour may constitute an additional source of VEGF. Elevated serum and ascitic fluid concentrations of VEGF has been detected in some cancer patients (Kondo et al., 1994, Boocock et al., 1995).

B. Wound healing:

In situ hybridisation studies have demonstrated an increase in VEGF mRNA expression in the hyperproliferative epithelium during wound healing. Growth factors present at the wound site such as, EGF, TGF- beta, TNF-alpha, and keratinocyte growth factor (KGF) mediate the process of wound healing by inducing VEGF mRNA expression (Frank et al., 1995).

C. Intraocular neovascularisation:

Studies have suggested that VEGF may be involved in subretinal angiogenesis in patients with ischaemic retinal diseases, such as diabetic retinopathy and retinal vein occlusion. Vitreous and aqueous concentrations of VEGF were found to be elevated in women with proliferative diabetic retinopathy and in non-age-related subretinal neovascularisation (Wells et al., 1996). It was observed that elevated progesterone concentrations during pregnancy worsened diabetic retinopathy by up-regulating intraocular VEGF concentrations (Sone et al., 1996).

D. Rheumatoid arthritis (RA):

Independent studies have suggested that VEGF may be involved in the pathogenesis of RA (Koch et al., 1994, Fava et al., 1994). VEGF concentrations within the synovial fluid were elevated in women with RA but not in women with other forms of arthritis. Furthermore, anti-VEGF antibodies significantly reduced the endothelial cell chemotactic activity of RA synovial fluid (Fava et al., 1994).

E. Psoriasis and other skin disorders:

Increased vascularity and permeability are characteristic of psoriasis. VEGF expression was found to be increased in psoriatic skin (Detmar et al., 1994), in epidermis over blisters and in the surrounding inflammatory infiltrates of skin disorders such as bullous pemphigoid, erythema multiforme and dermatitis herpetiformis (Brown et al., 1995).

F. Graves' disease:

Studies have shown that VEGF secreted by thyroid follicles, may be responsible for the characteristic hypervascularity of the thyroid in Graves' syndrome (Wartofsky, 1994).

Therapeutic applications:

Doppler blood flow studies in postmenopausal women have demonstrated increased peripheral vascular resistance after menopause (Gangar et al., 1991, Wren, 1993, Rozenburg et al., 1994). This putative adverse effect was reversed by treatment with oestrogens (Gangar et al., 1991, Wren, 1993, Rozenburg et al., 1994). Since VEGF production in the uterus is stimulated by oestrogens (Cullinan-Bove and Koos, 1993, Hyder et al., 1996), it was hypothesised that increased blood flow velocities in the carotid, uterine and other peripheral arteries in postmenopausal women receiving HRT may be the consequence of an increase in serum VEGF concentrations. The results of our study (Study 5, page 188, Agrawal et al., 2000) demonstrated that women who were treated with menopausal therapy had higher mean serum VEGF concentrations than women who were not on any menopausal therapy and treatment with conjugated equine oestrogens seemed to be the most potent stimulator of VEGF production. This study suggests that availability of agents to promote growth of new blood vessels may have a role in the treatment of postmenopausal women.

A. Inhibition of tumorigenesis:

The availability of specific monoclonal antibodies capable of inhibiting VEGF-induced angiogenesis in -vivo and in -vitro (Kim et al., 1992) has made it

possible to generate direct evidence for a role of VEGF in tumorigenesis. Such antibodies were found to exert a potent inhibitory effect (up to 70 to 95%) on the growth of tumour cells (Warren et al., 1995, Borgstrom et al., 1996). It is possible that inhibition of neovascularisation is the mechanism of tumour suppression (Kim et al., 1992).

B. VEGF induced angiogenesis:

The availability of agents to promote the growth of new collateral vessels would be of a novel therapeutic modality for disorders characterised by inadequate tissue perfusion and may constitute an alternative to surgical reconstruction procedures. Two such conditions are chronic limb ischaemia and coronary insufficiency.

B (i): Chronic limb ischaemia:

Chronic limb ischaemia is most frequently caused by obstructive atherosclerosis affecting the superficial femoral artery. It has recently been shown that intraarterial or intramuscular administration of human recombinant VEG_{165} or arterial gene transfer with cDNA encoding VEGF (Takeshita et al., 1996, a and b) significantly augmented perfusion and development of collateral vessels and muscle function (Walder et al., 1996) in a rabbit model where limb ischaemia was created by surgical removal of the femoral artery. Other angiogenic factors such as bFGF and TGF-$beta_1$ may have synergistic effects with VEGF in inducing ischaemic limb angiogenesis in-vitro (Asahara et al., 1995, Isner et al., 1996).

B (ii): Coronary insufficiency:

VEGF induces endothelium-dependent relaxation of coronary arteries, via the Ca^{2+} dependent synthesis and release of endothelium derived relaxing factor (nitric oxide) (Ku et al., 1993). Studies have shown that intraluminal administration of human recombinant VEGF within coronary arteries in a canine model (Banai et al., 1994) or extraluminal administration of human recombinant VEGF in a porcine model (Harada et al., 1996), with experimentally induced coronary insufficiency, increased coronary blood flow in these species. The benefit of such therapy has been demonstrated by

magnetic resonance imaging (Pearlman et al., 1995). A further potential therapeutic application of VEGF is the prevention of restenosis after percutaneous transluminal angioplasty (Callow et al., 1994, Asahara et al., 1995). Therefore human recombinant VEGF or VEGF gene therapy offers a new therapeutic modality for inducing angiogenesis in areas of arterial insufficiency which are usually refractory to conservative measures and unresponsive to pharmacological treatment.

Doppler ultrasonography:

In clinical studies in this thesis on the normal menstrual cycle and IVF treatment cycles, Doppler ultrasonography has been used as a supplementary investigative procedure to investigate the relationship of blood flow changes within uterine and ovarian blood vessels to changes in serum VEGF concentrations. Doppler ultrasonography is therefore described here briefly.

With the intervention of Doppler ultrasound, it has become possible to investigate vascular changes non-invasively. The development of pulsed Doppler systems allows the operator to sample signals at a chosen depth and, when combined with real-time ultrasound imaging ("Duplex method"), specific vessels can be precisely located and studied.

With the advent of transvaginal Doppler technique and the close proximity of the probe to the pelvic vessels, it became possible to locate accurately and describe flow characteristics of the uterine and ovarian arteries and the smaller vessels within the uterine and ovarian parenchyma (Kurjack et al., 1989). The introduction of colour Doppler ultrasound improved spatial evaluation and localisation of blood vessels. Furthermore colour Doppler ultrasound facilitates the detailed analysis of flow velocity waveform patterns by pulsed Doppler and also enables the operator to describe the subjective appearance of new vessel formation or "neoangiogenesis" (Bourne et al., 1989).

Blood flow changes to the uterus and/or ovaries during the spontaneous menstrual cycle in normal healthy women have been studied previously using Doppler technique (Taylor et al., 1985, Goswamy et al., 1988, Scholtes et al., 1989, Steer et al., 1990, Collins et al., 1991, Campbell et al., 1993, Sladkevicius et al., 1993, Zaidi et al., 1996). Transvaginal Doppler ultrasonography is also used nowadays to monitor ovarian function (Deutinger et al., 1989, Battaglia et al., 1990, Weiner et al., 1993, Balakier et al., 1995) and to assess uterine receptivity in IVF cycles (Steer et al., 1992, Tekay et al., 1995, Zaidi et al., 1995a., Zaidi et al., 1996). However it is only

recently that the technique has been used to study dysfunctional problems of ovarian function such as luteinised unruptured follicle syndrome (Zaidi et al., 1995a) and polycystic ovary syndrome (Battaglia et al., 1995, Zaidi et al., 1995).

The uterine artery originates from the anterior division of the internal iliac artery and is related laterally to the internal cervical os. It is here that the uterine artery can be visualised by conventional gray-scale ultrasonography.

As the ovarian artery enters the hilum it divides forming an anastomising arcade of vessels in the hilar region. In the ovarian cortex the vessels form vascular arcades in the stroma surrounding the follicles. During the development of an ovarian follicle, a rich irregular capillary plexus progressively develops in the theca surrounding the vascular granulosa cell layer of the ovarian follicle (Koos, 1989). These vessels can be seen by colour Doppler ultrasonography during the proliferative phase of the menstrual cycle (Sladkevicius et al., 1993, Tan et al., 1996). Following ovulation there is proliferation of the vessels of the theca, which merge to form the corpus luteum (Yen, 1986). Using colour Doppler the blood supply to the corpus luteum can be clearly seen as a bright coloured area within the corpus luteum (Sladkevicius et al., 1994, Zaidi et al., 1995d).

Principles of Doppler ultrasound:

In 1892 Christian Doppler demonstrated mathematically that the frequency of a sound wave appears to increase to an observer who is moving towards and to decrease to an observer who is moving away from its source. When a Doppler transducer emits a beam of ultrasound in the body, wave reflection from stationary tissues in the body are received at the emitted frequency. If the ultrasound waves are reflected from a moving target, the movement causes a frequency shift in the reflected signal. The ultrasound receiver detects this frequency shift (fd), also known as the Doppler shift frequency. This frequency shift is proportional to the velocity of the moving target and is expressed as follows:

$f_d = 2 f_t v \cos \equiv / c$

where f_t is the transmitted ultrasound frequency, v is the velocity of the target, c is the velocity of sound in the medium and $\equiv$ is the angle between the ultrasound beam and the direction of movement of the target.

Doppler shift information can be analysed qualitatively, quantitatively and semi-quantitatively.

Since the Doppler frequency shift is directly proportional to the flow velocity, with the constant of proportionality determined by the beam/vessel angle, the ratio of two Doppler shifted frequencies is independent of this angle. As a result it is possible to use such ratios as an index of pulsatility of the waveform and a measure of distal impedance. Several indices based on the maximum Doppler shift waveform have been described as a measure of this pulsatility.

A) Pulsatility Index (PI) was originally described by Gosling (1971) and is defined as:

PI = (S - D) / TAMXV

where S is the maximum Doppler shifted frequency, D is the minimum Doppler shifted frequency and mean or time-averaged maximum velocity over a cardiac cycle (TAMXV) is the mean of the maximum Doppler shift frequencies over the cardiac cycle.

B) Pourcelot's resistance Index (RI), also known as the resistance index, (Pourcelot, 1974), is defined as:

RI = (S - D) /S

where S is the peak systolic and D is the end diastolic frequency.

C) Systolic / Diastolic ratio is calculated by dividing the peak systolic height by the minimum diastolic height.

Each of these indices is independent of the beam/angle but the PI reflects blood flow impedance with most accuracy (Miles et al., 1987).

Doppler ultrasonographic assessment of ovarian and uterine arteries in healthy pre-menopausal women

Several Doppler ultrasound studies on uterine and ovarian blood flow have been published. Most have reported a rise in resistance indices (PI and RI) in the uterine arteries during the peri-ovulatory phase of the menstrual cycle (Goswamy et al., 1988, Battaglia et al., 1990, Steer et al., 1990, Collins et al., 1991, Sladkevicius et al., 1993) and a striking reduction of these indices in the early to mid-luteal phases (Goswamy et al., 1988, Battaglia et al., 1990, Steer et al., 1990, Kurjack et al., 1991, Sladkevicius et al., 1993). The uterine artery PI increases prior to the onset of menstruation, reaches its peak on the first day of menstruation and declines thereafter (Battaglia et al., 1990, Steer et al., 1990). The fall in uterine artery PI immediately after menstruation was confirmed by Sladkevicius et al., (1994).

Prior to 1991, studies on ovarian circulation concentrated on vascular changes within the ovarian artery. The validity of these studies is questionable, given the anatomical variation of the ovarian artery, difficulty in assessing it with ultrasound techniques and the near proximity of other pelvic vessels. Studies during this period reported PI values in the ovarian artery supplying the dominant follicle to be lower in the luteal phase than in the follicular phase of the menstrual cycle and lower than the PI of the ovarian artery of the nondominant ovary. No changes in PI were noted with the non-dominant ovary (Taylor et al., 1985, Scholtes et al., 1989, Schurz et al., 1990, Hata et al., 1990).

With the advent of improved ultrasound systems, studies have focused on much smaller intra-ovarian vessels (Bourne et al., 1991, Collins et al., 1991, Campbell et al., 1993, Sladkevicius et al., 1993, Kupesic et al., 1993, Sladkevicius et al., 1994, Zaidi et al., 1995b, 1995c, 1995d). Whilst studying vascular changes within the vessels surrounding the dominant follicle, it was found that peak systolic velocity (PSV) within these vessels rose on the day prior to ovulation (i.e. the day of LH surge) and continued to rise for three days after ovulation (Bourne et al., 1991, Collins et al., 1991, Campbell et al., 1993). Detailed analysis further demonstrated that the PSV within the vessels surrounding the dominant ovarian follicle rose with the growth of the

follicle, even prior to the preovulatory day, and continued to rise thereafter (Sladkevicius et al., 1993). Later, vascular changes in other branches of main arterial systems, e.g. radial arteries (Kupesic et al., 1993), spiral arteries (Sladkevicius et al., 1993), ovarian stromal arteries (Sladkevicius et al., 1993, Battaglia et al., 1995, Zaidi et al., 1995c, Bassil et al., 1997) and ovarian hilar arteries (Sladkevicius et al., 1993) were described.

Ultrasonography and PCOS:

PCO and ultrasonographic findings have previously been discussed. Although there are now clear ultrasound criteria for the diagnosis of PCO a number of difficulties remain. The follicular pattern and number of follicles are often difficult to measure accurately and stromal appearance and echogenecity remain a subjective parameter. Ultrasound features are dependent on probe frequency, beam attenuation, body habitus and gain settings. Despite this, the focus of ultrasound diagnosis of PCO has centered on stromal characteristics particularly since hyperandrogenaemia, a consistent feature of PCOS, appears to be ovarian in origin (Chang et al., 1983). Thus more objective and reliable ultrasound techniques for stromal assessment are needed particularly since stromal characteristics may be more important than absolute follicular number (Dewailly et al., 1994).

Recent developments include computerised two-dimensional ultrasound systems (Dewailly et al., 1994), three-dimensional ultrasound systems (Kyei-Mensah et al., 1998) and colour Doppler ultrasonography (Battaglia et al., 1995, Zaidi et al., 1995c). These studies have attempted to document ovarian stromal characteristics objectively and thus provide more reliable comparative studies between women with normal and polycystic ovaries. Dewailly et al., (1994) measured ovarian stromal and cyst area using two-dimensional ultrasound system in women with hyperandrogenism, hypothalamic anovulation and in normal controls. They showed that stromal area in the hyperandrogenaemic group was significantly greater than in women with normal androgen concentrations. Further, stromal area correlated positively with serum androstenedione and 17-hydroxyprogesterone concentrations thus linking, for the first time, a

quantitative stromal parameter with markers of androgenic dysfunction.

Recently, Kyei-Mensah and colleagues (1996), evaluated the Combison 530 three-dimensional ultrasound system (Kretztechnik AG, Zipf, Austria) which allows visualisation of the pelvis in the transverse plane, i.e. the plane parallel to the transducer face, in addition to the standard two dimensional longitudinal and transverse views. The area of interest can thus be captured and stored and the data used for later analysis. Using this system, Kyei-Mensah et al., 1998, reported differences in ovarian stromal volume in women with normal and polycystic ovaries and demonstrated that ovarian stromal volume is significantly greater in women with PCO than women with normal ovaries. Thus objective assessment of the ovarian stroma in this way may provide more reproducible diagnostic criteria (Fig 9.7.1 and 9.7.2).

Transvaginal colour and pulsed Doppler ultrasound in combination with real time ultrasonography is increasingly used as a non-invasive method to assess blood flow changes in the pelvic organs. Battaglia et al., 1995, assessed intraovarian and uterine artery indices of impedance to blood flow in women with PCOS and compared results with normal female volunteers. They observed significantly higher uterine artery PI and lower ovarian stromal resistance indices in women with PCOS compared with women with normal ovaries. They also demonstrated that obese women with PCOS had significantly higher uterine artery impedance compared with lean women with PCOS. Zaidi et al., (1995c) studied vascular changes within the ovarian stroma in three groups of women. Group 1 consisted of women with normal ovaries on ultrasound and had regular ovulatory menstrual cycles. Group 2 consisted of women similar to group 1, but had PCO on ultrasound examination. Group 3 consisted of patients with PCOS (as per previous definition). Significantly higher intraovarian perfusion was observed in women in groups 2 and 3. These studies suggest that there are significant differences in vascularisation between women with normal and PCO and may provide some insight into the different pathogenic mechanisms occurring in women with PCOS.

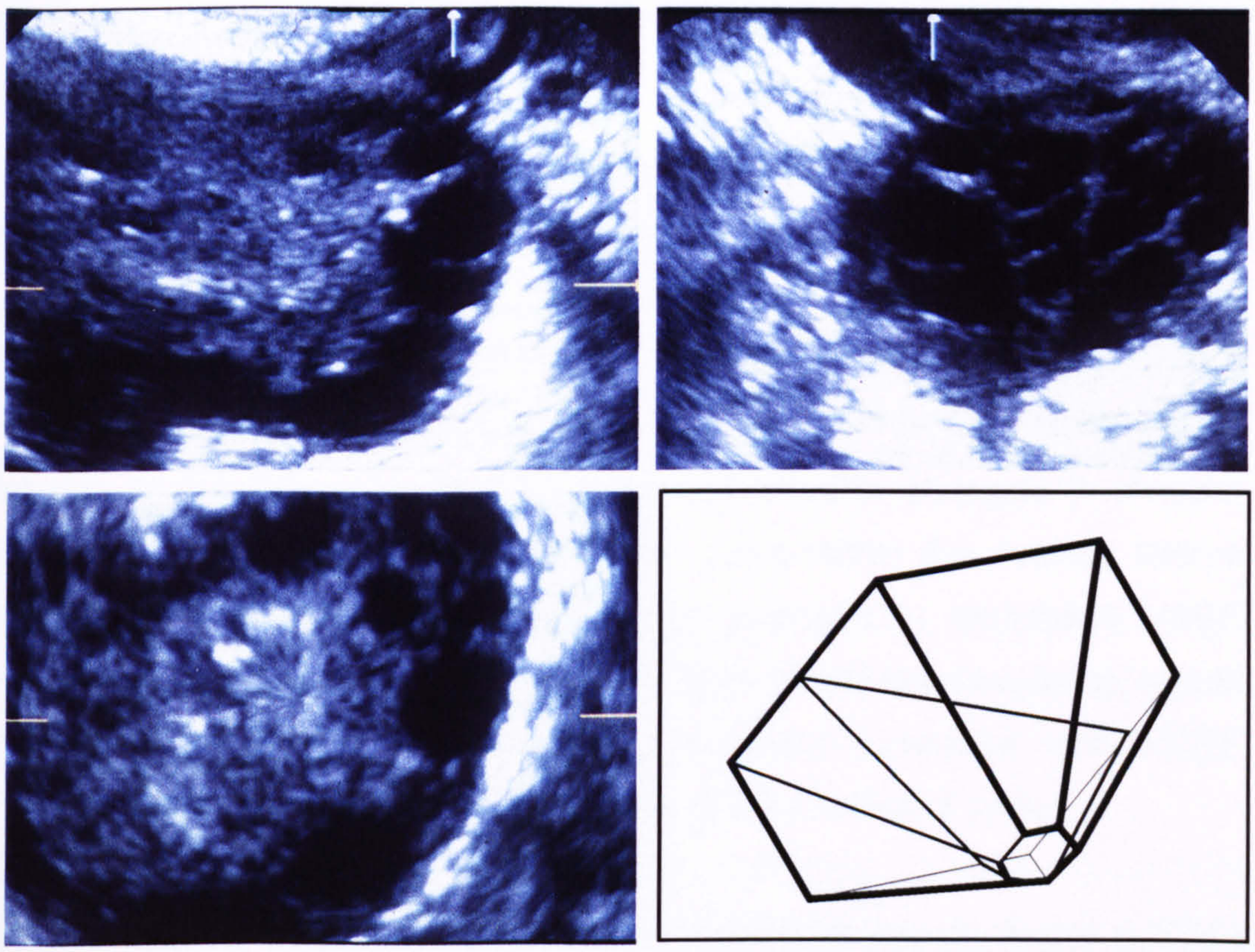


Figure 9.7.1

Three dimensional ultrasound image of a polycystic ovary in the early follicular phase of the menstrual cycle. The triplanar view gives a better indication of follicle number (Kyeimensah *et al*, 1995).

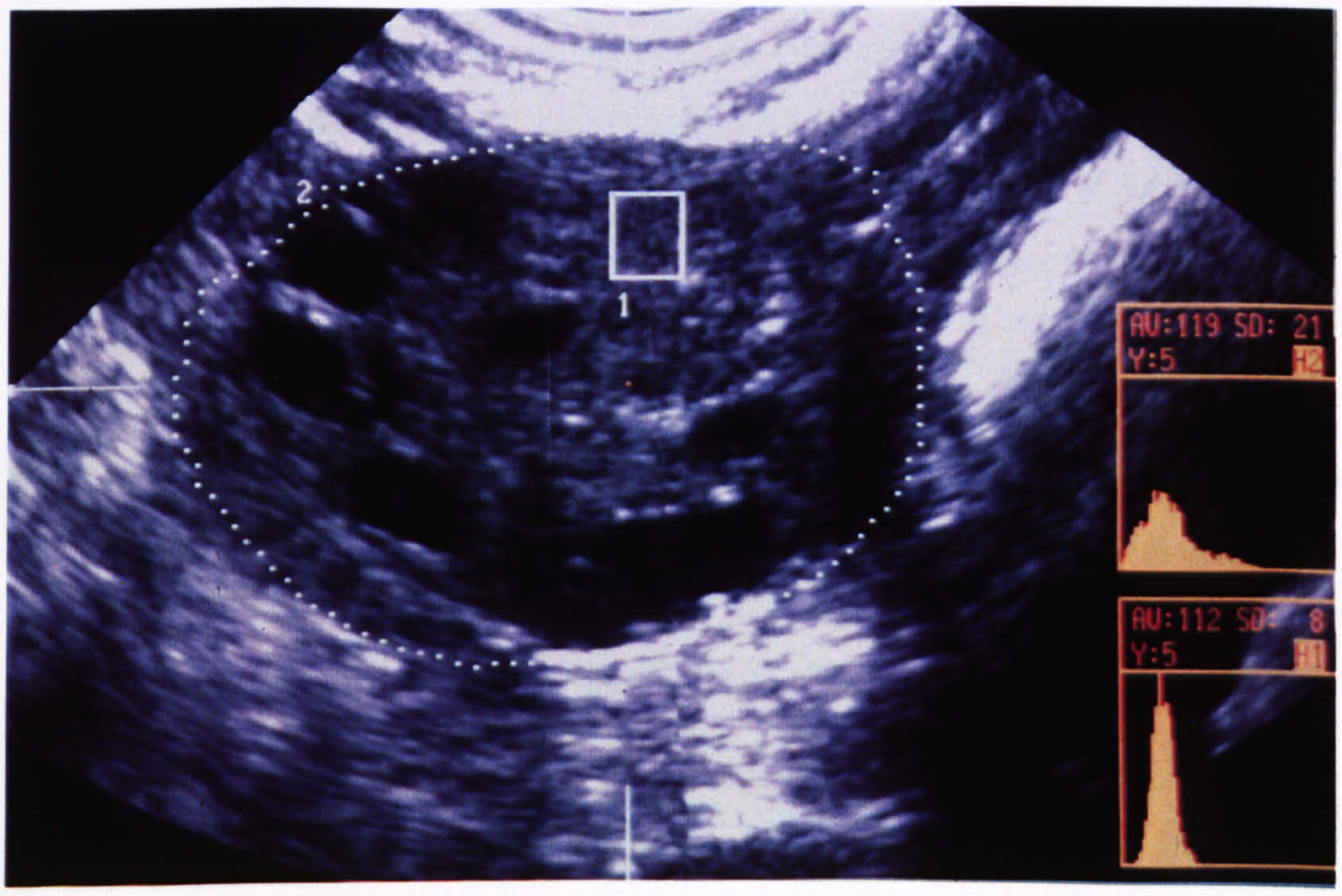


Figure 9.7.2

In a polycystic ovary, ovarian stromal volume is greater compared with ovarian follicular volume as measured by 3-D ultrasonography (Kyeimensah *et al*, 1995).

Aims:

AIMS:

Clinical studies:

Study 1: The study of serum vascular endothelial growth factor (VEGF) in the normal menstrual cycle and association with changes in ovarian and uterine Doppler blood flow in women and the study of serum VEGF concentrations in healthy men.

The aim of the first study was to determine whether changes in Doppler blood flow velocities during the menstrual cycle within the ovarian stroma and the uterine arteries are reflected by changes in peripheral VEGF concentrations in women. The aim was also to assess circulating serum VEGF concentrations in healthy male subjects compared with VEGF concentrations in women during all phases of the menstrual cycle.

Study 2: The study of serum vascular endothelial growth factor (VEGF) concentrations and ovarian blood flow in women with normal and polycystic ovaries.

The aim of the second study was to determine serum VEGF concentrations and Doppler blood flow changes within the ovarian stroma of women with PCO and women with normal ovaries and to establish whether there is a relation between serum VEGF concentrations and Doppler blood flow velocities within the ovarian stroma of women with normal and polycystic ovaries.

Study 3: The study of changes in serum vascular endothelial growth factor (VEGF) and Doppler blood flow velocities in ovarian and uterine blood vessels during IVF treatment cycles and its relevance to ovarian hyperstimulation syndrome and polycystic ovaries

The aim of the third study was to study whether the relationship of serum VEGF and blood flow changes within the ovarian stromal and uterine arteries, as assessed by pulsed and colour Doppler ultrasound, was present

throughout the in-vitro fertilisation (IVF) treatment cycle and whether they were related to the clinical occurrence of OHSS. I also aimed to establish serum and follicular fluid VEGF concentrations in women undergoing ovarian stimulation for IVF. A possible link between VEGF, OHSS and the presence of polycystic ovaries (PCO) was also explored.

Further, I aimed to observe whether serum VEGF concentrations differed in women who achieved pregnancy after IVF treatment from those who did not achieve pregnancy.

Study 4: The study of serum vascular endothelial growth factor (VEGF) in in-vitro fertilisation cycles as a predictor of the risk of ovarian hyperstimulation syndrome.

The aim of the fourth study was to explore the value of measurements of serum VEGF concentrations during IVF cycles compared with measurements of serum E_2, and follicle numbers on the day of hCG administration, oocytes retrieved on the day of egg collection and the presence of polycystic ovaries as a method of predicting ovarian over-response and, therefore, for preventing OHSS.

Study 5: The study of the effect of menopause and HRT on serum VEGF concentrations

In this study, the model of the menopause was used to estimate the contribution of the reproductive tract to circulating VEGF concentrations.

This cross sectional study was designed to assess VEGF concentrations in healthy postmenopausal women in relation to menopausal hormone replacement therapy. The aim was also to assess whether the presence or absence of a uterus in menopausal women, made any difference to serum VEGF concentrations.

Study 6: The study of the effect of hysterectomy and bilateral salpingo oophorectomy on serum VEGF concentrations.

The aim of this study was to study the effect of hysterectomy and bilateral salpingo oophorectomy on serum VEGF concentrations in pre-menopausal women.

Laboratory studies:

Study 7: The study of VEGF release by granulosa lutein cells cultured in-vitro.

a. The aim of the in-vitro studies was to determine whether release of VEGF by cultured luteinised human granulosa cells, could be demonstrated by in - vitro culture.

b. To investigate the effect of human recombinant hCG, insulin, FSH and LH on VEGF release by luteinised human granulosa cells.

c The hypothesis that raised serum VEGF in women with PCO/PCOS observed in clinical studies results from excess release by luteinised granulosa cells was tested by in-vitro studies. I therefore aimed to investigate whether there were any differences in VEGF released by luteinised granulosa cells obtained from women with PCO/PCOS compared with women with normal ovaries and in those obtained from women who developed OHSS compared with those who did not develop OHSS.

Material and Methods:

MATERIAL AND METHODS:

Section I) Subjects:

Study 1: The study of serum vascular endothelial growth factor (VEGF) in the normal menstrual cycle and association with changes in ovarian and uterine Doppler blood flow in women and the study of serum VEGF concentrations in healthy men.

Nineteen healthy volunteers at The London Women's Clinic with regular ovulatory menstrual cycles (range 28 -30 days) were recruited by myself (RA). Previous ovulation had been confirmed by measurements of serum progesterone in excess of 30 nmol/l). The subjects were not receiving treatment with the oral contraceptive pill. Those with a history or examination suggestive of endometriosis (n =2), uterine fibroids (n =2) or recent pelvic inflammatory disease (n =1) were excluded. The study group then comprised 14 women, 10 of whom were nulliparous. Ten women had normal ovaries and 4 had polycystic ovaries (PCO), as determined by ultrasonographic criteria (Adams et al., 1985, Fox et al., 1991). The mean age was 25 (range 23 to 31) years and mean ± SD body mass index (BMI) was 23.6 ± 2.8 kg/m^2.

In order to study serum VEGF concentrations in men, twelve healthy males who attended the sperm bank at The Middlesex Hospital, University College London, London, for sperm donation, were recruited. This study was done in collaboration with Dr. Ghulam Bahadur, The Chief of Sperm Bank Services at The Middlesex Hospital London. He was responsible for recruiting men in the study and blood sampling. These men had normal physical examination and had negative tests for gonorrhoea and chlamydia in the urethral swab. They had no history of familial genetic disorders. Semen samples were obtained, after they were screened negative for HIV, Hepatitis B and C, cytomegalovirus, VDRL/TPHA (tests for syphilis), and cystic fibrosis. Men with normal semen analysis and negative preliminary tests were recruited for the study.

Study 2: The study of serum vascular endothelial growth factor (VEGF) concentrations and ovarian blood flow in women with normal and polycystic ovaries.

Sixty consecutive women (46 nulliparous and 14 multiparous) women who attended The London Women's Clinic for IVF treatment were recruited by my colleagues Dr. Povilos Sladkevicius (PS), Dr. Lawrence Engmann (LE) and myself (RA). My colleagues PS and LE, were Research Fellows at The London Women's Clinic. These women had no detectable pelvic pathology, such as endometriosis, uterine fibroids and ovarian cysts. Their mean age was 35.4 years (range 25 to 43) and mean BMI was 23.8 $\pm$ 3.4 (SD) kg/m^2. The 60 women were divided into 3 groups according to the following criteria. The 'normal ovary group' (n = 36) had regular ovulatory menstrual cycles and had normal ovaries demonstrated on baseline ultrasound examination (day 2-3 of menstrual cycle). The 'PCO group' (n = 14) had regular ovulatory menstrual cycle and polycystic ovaries on ultrasound examination (Adams et al 1985, Fox et al 1991) but did not have clinical or biochemical evidence of polycystic ovarian syndrome (PCOS). The 'PCOS group' (n = 10) had PCO on ultrasound examination and a previous history of anovulatory menstrual cycles and/or oligomenorrhoea, with or without hirsutism, acne and obesity and/or elevated serum LH concentrations (≥10 IU/l) and/or elevated serum androgens in the early follicular phase.

Studies 3 and 4: The study of changes in serum VEGF and Doppler blood flow velocities in ovarian and uterine blood vessels during IVF treatment cycles and its relevance to OHSS and PCO and the study on serum VEGF during IVF cycles as a prediction factor for the risk of developing OHSS.

One hundred and seven women undergoing IVF between March and November 1996 at The London Women's Clinic, were recruited for the study by my colleagues PS, LE and myself. 65 women had normal ovaries, 27 had PCO and 15 had PCOS (Table 11.1). The patient's ages ranged from 27 to 47 years. Patients with detectable pelvic pathology such as endometriosis, uterine fibroids, ovarian cysts or pelvic inflammatory disease

were excluded from the study. The causes of infertility were male factor (56.8%), tubal disease (13.7%), unexplained (12.5%), mixed (16%) and others (2.6%).

Table 11.1. Demographic data of women with normal ovaries, polycystic ovaries and polycystic ovary syndrome.

Variable	Normal ovaries (n = 65)	PCO (n = 27)	PCOS (n= 15)	*P* value
Age (yrs)	36.3 ± 4.6*	34 ± 3.2	33 ± 3.7	*P* = 0.003
BMI (kg/m^2)	23.8 ± 3.4	23.7 ± 4.1	26.4 ± 4.1*	*P* < 0.05
Duration of infertility	5.2 ± 3.3	5.5 ± 3.2	6.8 ± 2.4	NS
Past attempts	1.8 ± 1.7	1.3 ± 1.4	1.8 ± 2	NS
No. who underwent IVF/ no. who underwent ICSI	46/24	13/14	11/4	NS
No. of parous women (%)	8 (18.4)	5 (18.5)	4 (26.6)	NS
No. with OHSS (%)	3 (4.6)	11 (40.7)**	6 (40)**	*P* = 0.0002
No. (%) with Infertility diagnosis Male factor	29 (44.6)	13 (48)	7 (46.6)	NS
Tubal factor	12 (18.4)	5 (18.5)	3 (20)	
Endometriosis	8 (12.3)	4 (14.8)	2 (13.3)	
Unexplained	6 (7.69)	2 (7.4)	1 (6.6)	
Mixed factors	10 (15.3)	3 (11.1)	2 (13.3)	
Basal FSH level (mIU/L)	7.2 ± 2.9	6.6 ± 3.8	6.2 ± 2.2	NS
Basal LH level (mIU/mL)	4.83 ± 2.3	5.5 ± 3	10.2 ± 2.9 †	*P* < 0.0001
Basal E_2 level (pmol/L)	149.3 ± 92	120.6 ± 67.8	166 ± 93.7	NS
Testosterone level (nmol/L)	0.82 ± 0.4	1 ± 0.5	1.5 ± 1.2 †	*P* < 0.05

*Age significantly higher in women with normal ovaries compared with women with PCO and PCOS.

*BMI significantly higher in women with PCOS compared with women with normal ovaries and PCO (ANOVA test).

** The number of women with PCO and PCOS who developed OHSS was significantly higher compared with women with normal ovaries who developed OHSS (Chi squared test).

† Basal serum LH and testosterone concentrations were higher in women with PCOS compared with women with normal ovaries and PCO (ANOVA test).

Note. All values are means ± SD unless otherwise indicated. NS = not significant.

Serum VEGF and Doppler blood flow changes were measured in IVF cycles of these 107 women.

Ten (9.3 %) patients developed mild OHSS, 7 (6.5%) developed moderate and 3 (2.8%) developed severe OHSS (13). In a post-hoc subdivision of the study, VEGF and blood flow changes were related to the presence or absence of OHSS. Women were divided into a "no OHSS group" (n = 87) and the "OHSS (mild, moderate or severe) group" (n = 20). There were no significant differences between these two groups with respect to age, duration of infertility (DOI), parity, previous IVF attempts, treatment received (IVF or ICSI), the infertility diagnosis, and the basal serum FSH, LH and oestradiol (E_2) concentrations. Basal serum testosterone concentrations were higher (1.54 ± 1.1 nmol/l vs. 0.87 ± 0.5 nmol/l) in women who developed OHSS, probably because of the higher proportion of women with PCOS in this group of women. Thus women who developed OHSS were more likely to have PCOS (85% vs. 29.8%, $p<0.005$) than those who did not.

In a further post - hoc subdivision of the study, VEGF and blood flow changes were related to the presence or absence of PCO. Here patients were divided into the normal ovary group (n = 65), PCO group (n = 15) and PCOS group (n = 10). There were no significant differences between these three groups with respect to duration of infertility (DOI), parity, past attempts, the treatment received (IVF or ICSI), the diagnosis and the basal serum FSH and oestradiol concentrations. Women with normal ovaries were however older compared with women with PCO and PCOS. Further analyses were therefore controlled for age. In addition women with PCOS had a significantly higher mean body mass index (BMI), mean basal serum LH and testosterone concentrations than women with normal ovaries and those with PCO (Table 11.1).

Blood samples were further obtained from 18 patients who were contactable and who achieved pregnancy 2 weeks post embryo transfer. Samples were also obtained in 18 patients who did not achieve pregnancy at a similar stage. These patients served as matched controls. All samples were obtained by myself.

In study 4, a further (post-hoc) analysis of women who developed OHSS in study 3, women were divided into the "no/mild OHSS" group (n=97) and the "moderate/severe OHSS" group (n=10). This analysis was performed to facilitate prediction of the clinically important form of OHSS (i.e. moderate and severe OHSS) since it is these two forms of OHSS, which are responsible for the distressing morbidity and occasional mortality associated with ovarian stimulation.

Study 5: The study of the effect of menopause and HRT on serum VEGF concentrations.

One hundred and ninety nine consecutive postmenopausal women who had been postmenopausal for more than five years were recruited for this cross-sectional study in collaboration with Dr. Gordana Prelevic, Senior Lecturer and Honorary Consultant at The Royal Free and The Middlesex Hospitals, London. Dr Prelevic was responsible for recruiting patients and obtaining blood samples. Women with a past history of endometriosis, ovarian cysts, uterine fibroids, neoplastic disease and deep venous thromboembolism were excluded. Forty women had received no menopausal therapy and 159 women had been on various forms of menopausal therapy for more than five years, without interruption. Women who received HRT with interruptions were excluded. 132 patients had their uterus in situ and 67 women had had a hysterectomy with or without bilateral salpingo-oophorectomy in the past. All women were assessed clinically and details of their gynecological history, smoking habits, alcohol consumption and the presence or absence of hypertension noted.

For analyses, women were divided in groups according to treatment regimens:

- Group 1 - 40 women had not received any menopausal therapy (6 had hysterectomy).
- Group 2 - 76 women received tibolone 2.5 mg daily (20 had hysterectomy) (Livial, Organon Laboratories, Cambridgeshire, UK)
- Group 3 - 49 women received transdermal oestradiol (24 women who had their uterus in situ also received sequential norethisterone acetate

(Estracombi ® or Estrapak ®, Ciba, Novartis Pharmaceuticals, Camberley, Surrey, UK) whilst 25 women who had had a hysterectomy with or without bilateral salpingo-oophorectomy received 50 μg per day of transdermal oestradiol alone (Estraderm TTS 50, Ciba, Novartis Pharmaceuticals)).

- Group 4 - 34 women received conjugated equine oestrogens (CEO) (18 women with uterus in situ received CEO 0.625 mg with sequential norgestrel 150 μg/day for 14 days per cycle (Prempak-C, 0.625 mg, Wyeth Pharmaceutical Laboratories, Maidenhead, Berkshire, UK) whilst 16 women who had had a hysterectomy received CEO only (Premarin, 0.625 mg, Wyeth Laboratories).

Study 6: The study of the effect of hysterectomy and bilateral salpingo oophorectomy on serum VEGF concentrations.

To study the effects of hysterectomy and bilateral salpingo oophorectomy on serum VEGF concentrations, 6 healthy premenopausal women were recruited undergoing hysterectomy and bilateral salpingo- oophorectomy and 6 women undergoing hysterectomy without bilateral salpingo-oophorectomy (age range 32 - 45 years) between April 1998 and April 1999 by our collaborators, Dr. Sanjula Sharma (Senior House Officer) and Dr. S Sathanandan, (Consultant Obstetrician and Gynaecologist) at The Harold Wood Hospital, Essex, UK. They recruited patients and obtained blood samples. Women with a past history of endometriosis, ovarian cysts, uterine fibroids, and neoplastic disease were excluded from the study. The indication for hysterectomy in each of these patients was dysfunctional uterine bleeding and prophylactic oophorectomy was performed in 6 women at patient's request to prevent the risk of developing ovarian cancer. There were no differences in the age (38 ± 11 and 35 ± 10 years), baseline mean serum FSH concentrations (7.9 ± 0.9 and 7.39 ± 0.4 IU/L), mean serum oestradiol concentrations (138 ± 12 and 143 ± 11 pmol/L) in women who had oophorectomy with hysterectomy and in women who did not have oophorectomy with hysterectomy nor the indication for hysterectomy between the two groups of women. None of the women was on hormonal

therapy prior to hysterectomy or 8 weeks after hysterectomy (Tables 12.11 and 12.12).

Section II) Methods:

II i) Treatment protocol for IVF:

Patients recruited for studies 2, 3 and 4 underwent IVF treatment. All patients had normal serum FSH concentrations in the early follicular phase of the menstrual cycle (<10 IU/L).

All patients followed a standard "long" protocol of pre-treatment with GnRH analogue, followed by stimulation with HMG or FSH for controlled ovarian stimulation (Tan et al., 1992). Pituitary desensitisation was achieved by treatment with buserelin acetate (Suprefact; Hoechst, Hounslow, UK) in a dose of 500 µg daily, administered subcutaneously for two weeks. The treatment with buserelin was started between the second or third day of the patient's menstrual cycle, once a normal baseline serum FSH concentration (microparticle enzyme immunoassay, MEIA; AxSYM.FSH; Abbott Labs, IL, USA) and a normal baseline ultrasound scan were confirmed. Pituitary desensitisation was confirmed by vaginal ultrasound using standard criteria of an endometrial thickness of less than 5 mm together with no ovarian cysts larger than 15 mm in diameter. Ovarian cysts larger than these were aspirated by the transvaginal route under ultrasound control. Patients with an endometrial thickness greater than 5 mm in depth were treated by administering a single dose of Gestone (Progesterone) 100 mg by intramuscular injection (Gestone; Paines & Byrne, West Byfleet, UK).

Ovarian stimulation was achieved using HMG (Pergonal; Serono Pharmaceuticals Ltd., Welwyn Garden City, Herts, UK or Humegon; Organon Labs Ltd., Cambs, UK), FSH (Metrodin-HP; Serono Labs or Orgafol; Organon Labs) or Normegon; Organon Labs). A dose of 150 IU of FSH was used in patients less than age of 35 years of age and in all women with polycystic ovaries. A dose of 225 IU was used in women greater than 35 years of age. After gonadotrophin administration was commenced, treatment with buserelin acetate was continued in a reduced dose of 200 µg by subcutaneous injection until the day of administration of hCG. Follicle

growth was monitored using transvaginal ultrasound. The dose of gonadotrophins was adjusted according to the results of ultrasound monitoring to achieve optimum follicular growth, the aim being to achieve at least three follicles of diameter greater than 18 mm and an appropriate serum oestradiol concentration (500 - 1000 pmol/l per mature follicle, measured by Immulite oestradiol immunoassay, Wales, UK). Human chorionic gonadotrophin (Pregnyl; Organon Labs) was administered in a dose of 5000 IU, if serum oestradiol was greater than 10,000 pmol/l and in a dose of 10,000 IU, if serum oestradiol concentration was less than 10,000 pmol/l.

Transvaginal oocyte retrieval was performed 36 hours after administering hCG, under ultrasound guidance, using intravenous sedation or general anaesthesia, if desired. Embryo transfer was performed 2 days after oocyte retrieval. Luteal support was given in the form of Cyclogest pessaries (Prog Ph EuR; Hoechst Roussel Ltd., Middx, UK) in a dose of 400 mg twice a day for a fortnight until the day of the pregnancy test. Oocytes were graded and their quality assessed and scored. The quality of the embryos was also graded on a scale of one to four. Surplus embryos were frozen and the outcome of the treatment cycle documented. A urine hCG test (Quickview 1 step, Medimar Laboratories, Wheatley, Oxford, UK) was performed 14 days after embryo transfer. If this pregnancy test was positive, luteal support was continued. A transvaginal ultrasound scan was performed two weeks after a positive test to confirm the presence of an intrauterine viable pregnancy. The pregnancy rate was defined as the proportion of women having a positive pregnancy test 2 weeks after embryo transfer and the clinical pregnancy rate as the proportion of women where a viable pregnancy has been determined by ultrasonography 4 weeks after embryo transfer. My colleagues Povilos Sladkevicius, Lawrence Engmann and myself performed all IVF treatment.

II ii) <u>Research Ethical approval:</u>

The Research Ethics Committee of The London Women's Clinic approved the first 4 clinical studies. The Research Ethics Committee of the University

College London Hospital and The Royal Free Hospital, University of London, approved the later studies. Written and informed consent was obtained from all subjects.

II iii) Pelvic Ultrasonography and Doppler ultrasound blood flow velocity measurements:

a) Ultrasound equipment:

All ultrasound examinations described in this thesis were performed using an Acuson 128 XP/10 ultrasound system (Acuson computed sonography 128XP/10 OB; Acuson Corp, Mountain View, CA, USA). Ultrasound examination was performed through the transvaginal route using an EV-519 endovaginal probe, which emitted an ultrasound frequency of 5 MHz for B mode imaging. The transducer had both colour and pulsed Doppler modes with a frequency of 5 MHz. The EV-519 had a scanning sector of 90^0 and a tilt aperture of 19 mm. The focal range of the transducer could be varied easily according to the depth of the organ studied. The Doppler system was equipped with a high-pass filter to remove signals from vessel wall movements in the path of the Doppler ultrasound pulse. In the studies described, the high-pass filter was set at 125 Hz so that low blood flow velocities were detected. The spatial peak time average intensity (SPTA) of ultrasound for B-mode and Doppler examinations was <50 m W/cm^2, which is well within the "safety limits" recommended by the Bioeffects Committee of the American Institute of Ultrasound in Medicine (Gill, 1982).

b) Examination technique:

Gray-scale ultrasound examination:

Transvaginal ultrasound examinations were performed on all patients with empty bladders using the Trendelenberg position. The endovaginal probe was covered with a coupling gel and a sterile condom. The uterus was initially examined by standard techniques (Steer et al., 1990). Uterine morphology was confirmed normal in all subjects. The maximum endometrial thickness was measured as the maximum distance between each myometrial / endometrial interface through the central longitudinal axis of the uterus (Fleischer et al., 1986). Ovarian size was measured at the

baseline ultrasound scan in three orthogonal diameters and calculated from the length, depth and the width of the ovary. Follicular size was similarly measured thus allowing mean follicular diameter to be calculated. Follicle rupture was determined according to standard ultrasonographic criteria (Dewbury et al., 1993).

Pulsed and colour Doppler ultrasonography:

The vascularisation of the pelvic organs was visualised using this technique and blood flow velocity waveforms were obtained by placing the Doppler range gate over the coloured areas and activating the pulsed Doppler function. The Doppler range gate determines the location from which Doppler information is processed and displayed.

a) *Uterine artery*

The uterine artery was visualised by placing the colour Doppler box over the region of the internal cervical os with the probe in a longitudinal plane. The transducer was directed into each vaginal fornix, thereby visualising the uterine artery ascending up the lateral aspect of the uterus. The angle of insonation was adjusted to obtain maximum colour intensity. The pulsed Doppler range gate was then applied across the vessel, ensuring that the angle between the Doppler beam and the vessel was as close to 0° as possible. Flow velocity waveforms illustrating the shifted Doppler frequencies were thus demonstrated.

b) Ovarian stromal vessels:

Blood vessels could be clearly visualised when the colour Doppler box was placed over the ovarian stroma. An ovarian stromal artery was defined as any small artery in the ovarian stroma, not close to the surface of the ovary or near the follicle wall. Areas of maximum colour intensity, representing the greatest Doppler frequency shifts, were selected for examination. These intraovarian vessels were generally small. No correction was made for the angle of insonation but the highest achievable Doppler shift signals were sought in the ovarian stroma.

As the velocity recorded by Doppler ultrasound is a vector of blood flow velocity, it has to be corrected for the angle of insonation. The higher the

angle of insonation, the greater the effect of angle error on the resulting blood flow velocity value and vice versa. Clearly it is impossible to measure the insonation angle in these small vessels. It can however be assumed that at least one vessel in the vascular bed will be located at an appropriate angle so that maximum peak velocities can be measured with minimum error (Sladkevicius et al., 1993). Furthermore, at each examination the highest possible Doppler shift with the smallest possible angle was obtained, thereby allowing comparison of blood flow velocities between groups and within groups to be done.

c) Follicle vessels:

Ovarian stromal vessels terminate as vascular arcades around the wall of the developing ovarian follicles. They were seen circumferentially around the developing follicle. These vessels probably represent an anastomotic network of capillaries surrounding the avascular granulosa cell layer. The technique of selecting the flow velocity waveforms was similar to that described for the ovarian stromal vessels.

Blood flow velocity changes within the main ovarian artery were not studied because this vessel is extremely difficult to locate and recordings are associated with large errors (Scholtes et al., 1989, Kupesic and Kurjak, 1993).

c) Blood flow velocity waveform analysis:

In all studies, baseline measurements of peak systolic velocity (PSV) and time averaged maximum velocity (TAMXV), (measurements of blood flow velocity) and the pulsatility and resistance indices (PI and RI) (measurements of the resistance of blood flow) were made both on ovarian stromal blood vessels and on the uterine arteries at the beginning of the menstrual cycle (day 2 or 3, early follicular phase of the menstrual cycle).

All examinations were performed before midday by my colleagues, Povilos Sladkevicius and Lawrence Engmann and myself, to reduce the effects of diurnal variation in blood flow (Zaidi et al., 1995). Blood flow images were

recorded on videotape and stored for later analysis.

In addition in study 1, daily ultrasound examinations were performed from the 11th day of the menstrual cycle for tracking the growth of the dominant follicle. Further measurements of Doppler ultrasound blood flow were done once the dominant follicle reached 18 -20 mm in diameter. A blood sample was obtained to measure serum concentrations of LH and VEGF on this day. Twelve subjects had a LH surge on the day of the examination and two the following day (confirmed by serum LH surge). Doppler blood flow, serum VEGF and hormone measurements were repeated again seven days after ovulation. As observed by vaginal ultrasound examination, each woman developed a dominant follicle and subsequently a corpus luteum, accompanied by a midluteal serum progesterone concentration in excess of 30 nmol/l. I (RA), made all observations. The intra-observer coefficient of variation was less than 10% for TAMXV and PI (data not shown).

In study 2, all examinations were performed at the beginning of a menstrual cycle (day 2 or day 3) as stated earlier, prior to starting IVF treatment. Colour flow mapping and pulsed Doppler measurements were performed on ovarian stromal blood vessels once normal pelvic findings were confirmed. Blood flow velocity waveforms were thus detected and recorded. The PSV, TAMXV, PI and the RI were assessed within both ovaries.

My colleagues, Povilos Sladkevicius and Lawrence Engmann and I, made all observations. The interobserver coefficient of variation for TAMXV was 24% and for PI was 14%, as previously described (Sladkevicius et al 1993). The intraobserver coefficient of variation was less than 10% for TAMXV and PI.

In studies 3 and 4, PSV, TAMXV, PI and the RI were measured within both the ovarian blood vessels and the uterine arteries. In all patients observations were made in the early follicular phase of the menstrual cycle (day 2 or day 3) prior to starting IVF treatment, after pituitary desensitisation with buserelin and on the day of hCG administration. In 18 patients Doppler blood flow measurements were also recorded on the day of oocyte retrieval,

i.e., 36 hours post hCG, and on the day of embryo transfer. My colleagues, Povilos Sladkevicius and Lawrence Engmann and I, made all observations.

II iv) <u>Measurements of serum and follicular fluid VEGF concentrations:</u>

In studies 1, 2, 3 and 4, blood samples for serum VEGF and hormone measurements were obtained between 0800 h and 1200 h immediately after the ultrasound examination by staff nurses at The London Women's Clinic or myself. Blood samples from men were obtained at the time of semen analysis at The Middlesex Hospital. I centrifuged all blood samples within 40 minutes of obtaining them and the serum thus obtained was stored at -70°C for subsequent analysis. Follicular fluid obtained by myself for study 3 and 4 was also stored after centrifugation, at -70°C until analysis.

In study 1, blood samples were obtained in the early follicular phase (Day 2-3) of the menstrual cycle, on the day of LH surge and in the midluteal phases of the menstrual cycle.

In study 2, blood samples were obtained from women undergoing IVF on Day 2-3 of the menstrual cycle (i.e. at the beginning of the IVF treatment).

In studies 3 and 4, blood samples were obtained in all patients in the early follicular phase of the menstrual cycle (day 2 or day 3) prior to starting IVF treatment, after pituitary desensitisation with buserelin, on the day of hCG administration, oocyte retrieval and embryo transfer. Twenty women who developed OHSS and 10 women who did not develop OHSS had blood samples also taken, 3, 5 and 7 days after embryo transfer. Follicular fluid obtained during oocyte retrieval was also analysed for VEGF concentrations. Eighteen patients who achieved pregnancy had serum samples obtained at 4 weeks of gestation (2 weeks post embryo transfer). Serum samples were also obtained from 18 women who did not achieve pregnancy during similar periods. These women served as controls.

In study 5 fasting blood samples were taken for serum VEGF measurements and serum stored until analysed.

In study 6 blood samples were obtained when patients attended the hospital prior to hysterectomy at a convenient time and at 8 weeks after hysterectomy. None of the women was on HRT at any stage.

VEGF assays were performed by Mrs. Nadia Payne who is the Chief Laboratory Technician at the Cobbold Laboratory located in The Middlesex Hospital, London. Some assays, which were performed by me, were under her direct supervision.

Cytokit Red™ enzyme immunoassay

For studies 1 to 7, serum VEGF concentrations were measured using enzyme immunoassay (Cytokit Red™ EIA kits, Cyt Immune Sciences, Inc., Peninsula Labs, Inc., College Park, MD, USA). This in - vivo enzyme immunoassay kit detects "total" human VEGF in biological fluids (e.g., serum, plasma, CSF, saliva, urine) and cell culture supernatants. "Total" circulating VEGF represents the 'free' and 'bound' form of variant $VEGF_{165,}$ which is the major circulating form of VEGF. The 'bound' form of VEGF is usually bound to plasma proteins (e.g., $alpha_2$ macroglobulin, Houck et al., 1992). The major circulating forms of VEGF are $VEGF_{121}$ and $VEGF_{165,}$ while $VEGF_{189}$ and $VEGF_{206}$ are largely bound to cell membranes and do not represent measurable VEGF (Houck et al., 1992, Anthony et al., 1997, Heney et al., 1995, Soker at al., 1993).

Each kit has enough reagents for two 96-well immunoassay plates. Duplicate wells were read for sample and standards. A total of 82 samples could be analysed in duplicate with each kit. The assay time was 4 hours. The assay sensitivity was 0.195 ng/ml (9 pg/well). The detectable range for VEGF was 0.195 - 200 ng/ml, i.e., 9 - 10,000 pg/well.

The intra-assay coefficient of variation was 7.8% and the inter-assay coefficient of variation was 12.2%. The cross reactivities of the assay were <0.5% against WHO cytokine standards: (Human IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IL-10, TNF alpha, GM-CSF, G-CSF and M-CSF. The assay was validated by parallelism and quantitative recovery. The kit was stored until used, at 4^0 C and the assay performed at room temperature.

Cytokit Red ™ VEGF is a "competitive" enzyme immunoassay (EIA), which measures natural and recombinant forms of $VEGF_{165}$.

Principle of Cytokit Red ™

In this system, wells pre-coated with goat anti-rabbit antibodies are used to capture a specific cytokine complex in each sample, which consists of cytokine monoclonal antibody, biotinylated cytokine, and sample / standard. Cytokine specific antibody, biotinylated cytokine conjugate (competitive ligand), and sample or standard form a competitive reaction in which the samples or standards compete for antibody binding sites with the biotinylated cytokine. Therefore, as the concentration of cytokine in the sample increases, the amount of biotinylated cytokine captured by the antibody decreases. With the addition of Streptavidin - conjugated alkaline phosphatase (which binds only to the biotinylated cytokine), substrate solution and the amplifier solution the amount of biotinylated cytokine can be detected (Fig 11.2.1). There is an inverse relationship between optical density (OD) and concentration, that is, the higher the OD the less cytokine in the sample.

Colour generation system:

The kit uses an amplified colour generation system in which alkaline phosphatase reaction provides a cofactor that initiates a redox cycling reaction, leading to the formation of a coloured red (formazan) product. In this system, alkaline phosphatase dephosphorylates the reduced form of NADPH (substrate) and is converted to reduced NADH. The NADH then serves as a specific cofactor that activates another redox cycle driven by the amplifier which is the secondary enzyme system consisting of alcohol dehydrogenase and diaphorase.

Kit materials:

Each kit contains:

- Wash buffer: One 100 ml bottle of 20x concentrate wash buffer.
- Assay diluent: One 75 ml bottle of 1x concentrate assay diluent.
- VEGF antibody: Two vials of lyophilised VEGF antibody.
- VEGF standard: Two vials of lyophilised VEGF standard.

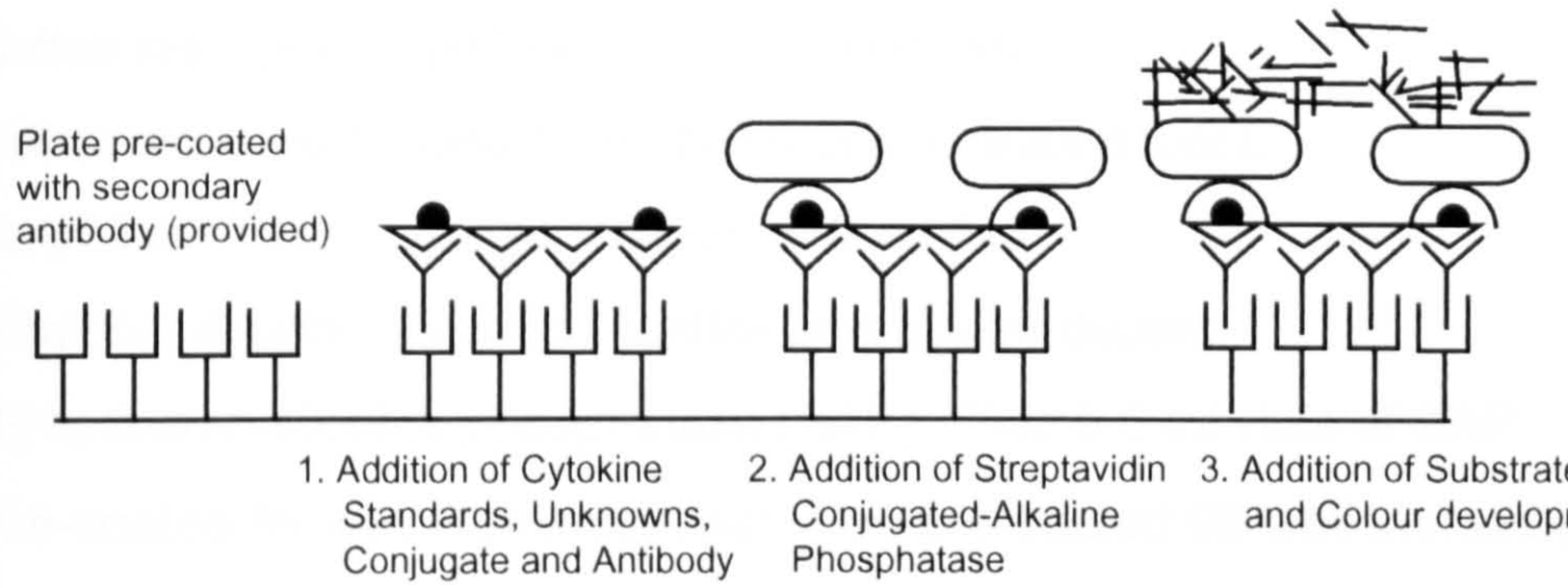
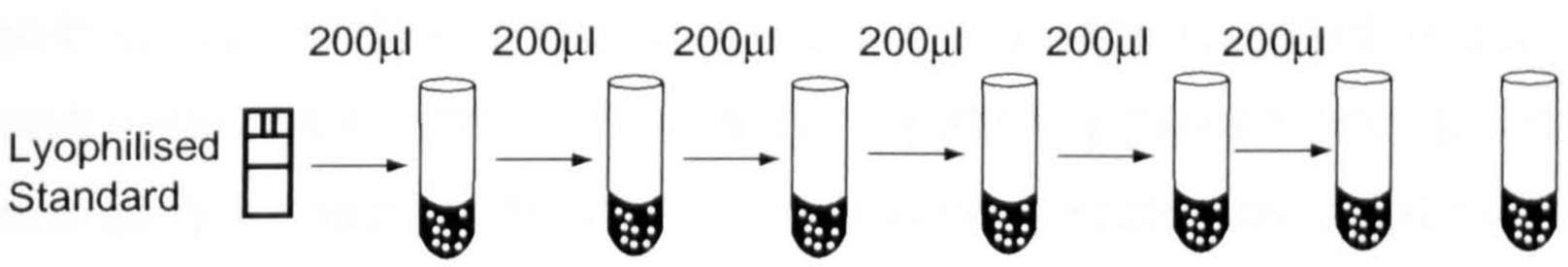


Figure 11.2.1

Schematic representation of the principle of the Cytokit Red™ competitive enzyme immunoassay.



Initial Volume (ml):

625	600	600	600	600	600	600	600

Concentration (ng/ml):

800	200	50	12.5	3.125	0.8	0.19	0.0

Standard Number:

#1	#2	#3	#4	#5	#6	"0 Dose"

Figure 11.2.2

Serial dilutions of serum samples in Cytokit Red™ enzyme immunoassay. (Figures 11.2.1, 11.2.2 and 11.2.3 reproduced from information leaflets supplied in the Cytokit Red™ immunoassay kit).

- VEGF conjugate: Two vials of lyophilised VEGF conjugate.
- Serum diluent: One 15 ml bottle of serum diluent.
- Substrate: One lyophilised bottle of substrate.
- Substrate diluent: One 13 ml bottle of substrate diluent.
- Amplifier: Two lyophilised bottles of amplifier.
- Amplifier diluent: Two 8 ml bottles of amplifier diluent.
- Streptavidin-alkaline phosphatase (SAP): Two 0.5 ml vials of SAP.
- Pre-coated 96 well immunoplates: Two pre-coated 96 well immunoplates sealed in foil pouches.
- Acetate plate sealers: Four acetate plate sealers sealed in foil pouches with the immunoplates.

Separate vials of cytokine antibody, cytokine standard, cytokine conjugate, SAP, amplifier and amplifier diluent, were provided for each immunoplate. However, only one bottle each of serum diluent, wash buffer, assay diluent, substrate and substrate diluent were provided with sufficient volumes to use on both immunoplates. Other materials which were required in addition to the one mentioned were, multi-channel pipettes, pipettors and tips capable of accurately measuring 10-1000 µl, graduated serological pipettes (10 and 25 ml), 96 - well microtitre plate reader with 490 nm filter, semi-log graph paper for manual plotting of data, 6, 15 and 50 ml test tubes, mechanical vortex and one litre container.

<u>Preparation of reagents:</u>

1) *VEGF antibody:*

a: The bottle of assay diluent was vortexed and 3 ml was added to a 15 ml conical screw-cap tube.

b: The lyophilised VEGF antibody was reconstituted with 1 ml of assay diluent.

c: 800 µl of reconstituted VEGF antibody was added to the 3 ml of assay diluent in the 15 ml tube and vortexed.

2) *VEGF standard:*

a: 7 test tubes were labelled from 0 to 6. 66 µl of serum diluent was added

to each of the tubes.

b: The lyophilised VEGF standard was reconstituted with 625 µl of serum diluent and vortexed. This solution had a concentration of 800 ng/ml.

c: 200 µl of the reconstituted VEGF standard from the vial was added to tube 1 and vortexed. This was standard 1 with a concentration of 200 ng/ml.

d: Standards 2 to 6 were then prepared by performing a 1:4 dilution of the preceding standard (Fig 11.2.2), (e.g., to make standard 2, 200 µl of standard 1 was added to tube 2 and vortexed and so on). VEGF standard was not added to the "0 dose" standard tube.

3) VEGF conjugate:

a: 3 ml of assay diluent was added to a 15 ml conical screw cap tube.

b: The lyophilised VEGF conjugate was reconstituted with 1 ml of assay diluent and vortexed.

c: 600 µl of reconstituted VEGF conjugate was added to the 3 ml of assay diluent in the 15 ml tube and vortexed.

4) Diluting Wash buffer:

The entire contents of concentrated wash buffer were diluted to 2 litres with deionised water. It was stirred to homogeneity.

5) Streptavidin - Alkaline Phosphatase (SAP):

The vial of SAP was vortexed and 400 µl was added to 12 ml of assay diluent and vortexed.

6) Substrate solution:

This was prepared after step 7 of the assay procedure, which has been described later.

a: The reagents were brought to room temperature.

b: The lyophilised substrate was reconstituted 10 minutes before use by adding the entire contents of the substrate diluent.

c: The mixture was gently mixed until completely dissolved. The bottle was resealed with a screw-top cap and not left uncovered.

7) Amplifier Solution:

This was prepared after step 7 of the assay procedure, which has been described later.

a: Reagents were brought to room temperature.

b: The lyophilised amplifier was reconstituted 10 minutes before use by adding the entire contents of one bottle of amplifier diluent to one bottle of lyophilised amplifier.

c: The mixture was gently mixed until completely dissolved. The bottle was resealed with a screw-top cap and not left uncovered.

Storage:

Approximately 5 ml of substrate and amplifier solution was required for each immunoplate. If the substrate solution was used on multiple occasions, the unused portion was stored in appropriate aliquots at -20 ^{0}C for a maximum of 30 days. Extended storage of other diluted solutions was maintained at 4 ^{0}C for no more than 14 days.

Assay procedure:

1) A standard curve was run at each assay procedure by dispensing 100 µl of the "0 dose" and standards "1 to 6" into the designated wells.

2) The serum samples to be analysed were diluted 1:2 with assay diluent. 50 µl of each sample and 50 µl of assay diluent were added to each well to make a total volume of 100 µl. For tissue culture samples 100 µl of each sample was added to their designated well.

3) 25 µl of diluted VEGF antibody was added to each well.

4) The plate was sealed with acetate plate sealer to prevent evaporation and incubated at room temperature for 3 hours.

5) The plate sealer was then removed and 25 µl of VEGF conjugate was added to each well . The plate was resealed and incubated at room temperature for 30 minutes.

6) Wash step: The plate sealer was removed and the plate washed 5 times. Plate washing is an important step and was done meticulously. A multichannel pipette was used to fill each well with 250 µl of diluted wash buffer. Fluid removal from the wells was best accomplished by inverting the

plate over a sink and flicking the fluid out of the wells and then forcefully blotting the plate on clean paper towels. After the last wash, each well was aspirated to remove any excess fluid that remained.

7) 50 µl of the diluted SAP was dispensed in each well. The plate was resealed and incubated at room temperature for another 30 minutes. The colour generation reagents were allowed to come to room temperature. After 20 minutes the substrate and amplifier solutions were prepared.

8) The plate sealer was removed and the plate was washed again 5 times as described in step 6.

9) 50 µl of the prepared substrate solution was dispensed in each well. The plate was resealed and incubated at room temperature for 20 minutes.

10) 50 µl of the prepared amplifier solution was dispensed in each well in the same order as in step 9. The plate was read 5 to 10 minutes after adding amplifier solution.

If the plate was not adequately washed, colour developed very rapidly and there were no gradations in colour in the standard curve (i.e. Optical Density for the entire plate reached 2.0 in less than 5 minutes). If this occurred, the plate was washed and steps 9 and 10, repeated.

11) Reading the plate: The plate reader was set to read at 490 nm. The reader was not blanked at the "0 dose". The plate was incubated at room temperature and the initial reading taken at 5 - 10 minutes after adding amplifier solution. The plate was read at 5 minutes intervals to establish a reference point. The plate was read continuously until the Optical Density for the "0 Dose" reached between 1.5 and 2.0.

<u>Calculation of results:</u>

Manual plotting:

The standard curve was plotted on semi-log graph paper (Fig 11.2.3). Known concentrations of VEGF were plotted on the log scale (X-axis) and the corresponding OD on the linear scale (Y-axis). The standard curve had a sigmoid curve that showed an inverse relationship between VEGF concentrations and the corresponding OD's (absorbances). Therefore, the greater the concentration of VEGF in the sample, the, lower the OD, or the

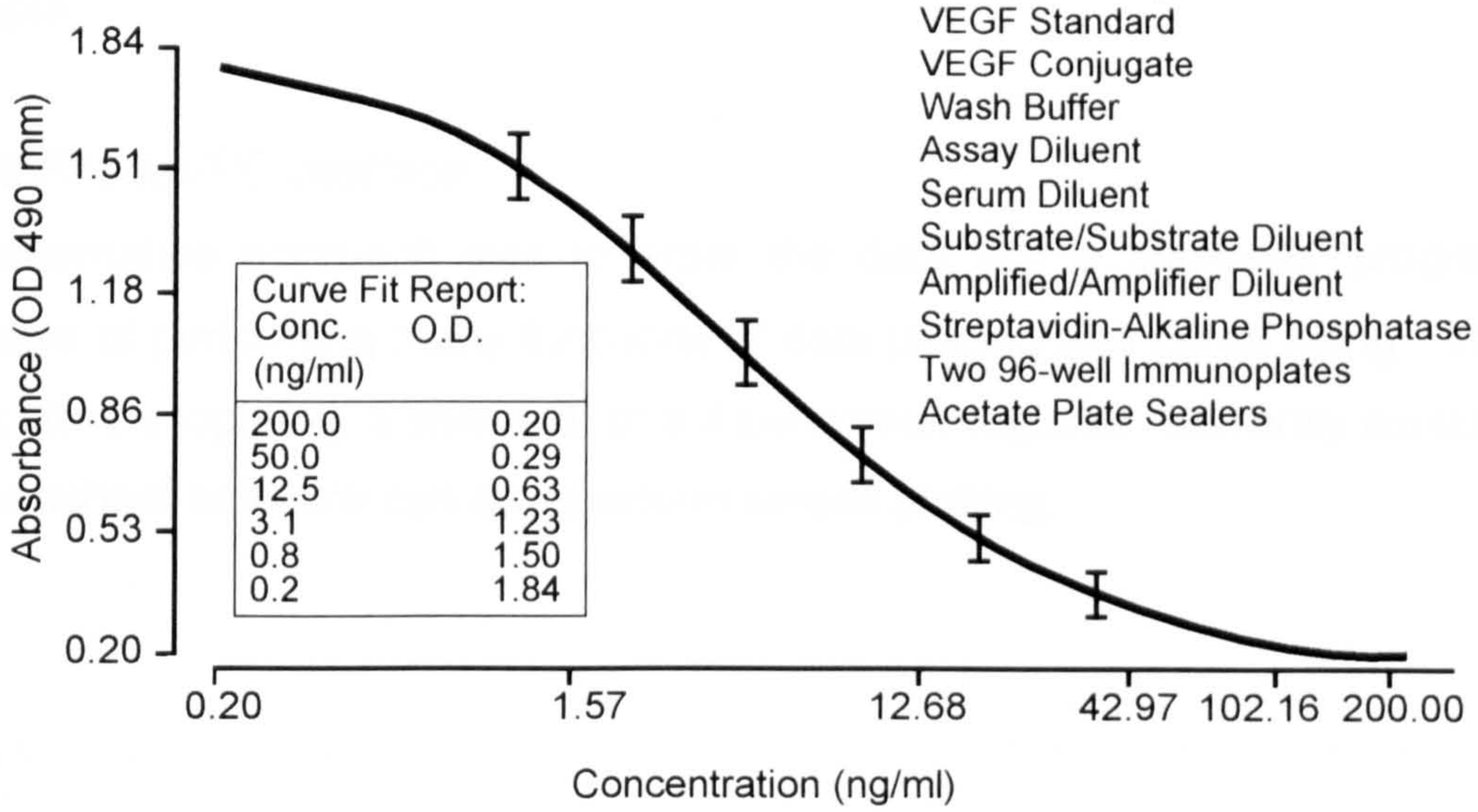


Figure 11.2.3

Cytokit Red™ VEGF Standard Curve.

less the red colour. The concentration of VEGF in unknown samples was determined by plotting the sample OD on the Y-axis, by drawing a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersected the X - axis at the concentration of VEGF in the unknown sample.

Plate Reader/PC interface:

An alternative approach was to enter the data into a computer program capable of performing many functions of data plotting and curve fitting. The data fitted a logit-log, a spline fit, or a 4 parameter logistic. Currently existing spreadsheet software can also perform simple plotting.

Effect of time, temperature and blood sample type on VEGF measurements by Cytokit immunoassay

In order to explore the effect of time and the temperature of storage of blood samples (i.e., establish a time and temperature response curve) and the type of blood samples obtained for VEGF measurements, the following experiment was conducted.

I obtained blood samples from 6 women in the early follicular phase of the menstrual cycle. Three patients had PCO and 3 had normal ovaries. There were no differences in the characteristics of these women (data not shown).

Blood samples were obtained in plain bulbs for separation of serum, in heparin bulbs for separation of heparin plasma and in EDTA bulbs for separation of EDTA plasma. 100 µl of each sample (i.e., serum, heparin plasma and EDTA plasma) were stored, each in 13 aliquots. One aliquot of each sample was analysed within 1 hour for a fresh assay. 4 aliquots of each sample type were stored at 4^0 C, -20^0C and at -70^0 C. One aliquot stored at each of the temperatures was then analysed for VEGF concentrations at 2, 4, 12 and 24 weeks post storage. The recovery of VEGF is shown in the "results" section.

Quantakine ™ human VEGF immunoassay:

For study 7, (the granulosa cell culture study), VEGF in cell culture supernatant was measured using this assay. Although the Cytokit Red™ VEGF enzyme immunoassay detected serum VEGF with adequate precision and sensitivity, VEGF in cell culture supernatants could not be detected. The Quantakine immunoassay (R&D systems, Inc., N.E., Minneapolis, MN, USA) was therefore tested for measuring VEGF concentrations in culture media and used successfully.

The Quantakine VEGF immunoassay is a 4.5 hours solid phase ELISA designed to measure $VEGF_{165}$ concentrations in cell culture supernatants, serum and plasma. It uses insect cell *Sf* 21- expressed recombinant human $VEGF_{165}$ and a polyclonal goat antiserum raised against the recombinant protein. Results obtained for naturally occurring human VEGF and recombinant human $VEGF_{121}$, showed linear curves that were parallel to standard curves obtained using the Quantakine kit standards. The results indicate that this kit can be used to determine relative mass values for natural human VEGF.

The detection of mRNA encoding a soluble VEGF receptor, Flt-1, suggests that this receptor may be present in some biological fluids. Soluble receptors present in biological samples do not necessarily interfere with the measurement of their corresponding ligands in these samples. However, the possibility of the interference of this receptor in VEGF measurements using this assay cannot be excluded. The predicted effect would be a lowering of the apparent VEGF content measured.

Principle of the assay:

This assay employed the Quantakine "sandwich enzyme immunoassay" technique. A microplate was pre-coated with a monoclonal antibody specific for VEGF. Standards and samples were pipetted into the wells and VEGF present, was bound by the immobilised antibody. After washing away any unbound substances, an enzyme-linked goat polyclonal anti - rabbit antibody specific for VEGF was added to the wells. Following a wash to remove any

unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of VEGF bound in the initial step. The colour development stopped spontaneously 20 - 30 minutes later. The intensity of the colour was measured (described later).

Reagents and kit materials:

- VEGF microplate: 96 well polystyrene microplate (12 strips of 8 wells) coated with a murine monoclonal antibody against VEGF.
- Wash buffer concentrate: 21 ml of a 25 fold concentrated solution of buffered surfactant with preservative.
- VEGF standard: Three vials (2000 pg/ml) of recombinant human VEGF in a buffered protein base with preservatives, lyophilised.
- VEGF conjugate: 21 ml of polyclonal antibody against VEGF conjugated to horseradish peroxidase, with preservatives.
- Assay diluent: 11 ml of a buffered protein base with preservative.
- Calibrator diluent RD5K - 21 ml of a buffered protein base with preservatives (For cell culture samples).
- Calibrator diluent RD6U: 21 ml of animal serum with preservatives (For serum and plasma).
- Colour reagent A: 12.5 ml of stabilized hydrogen peroxide.
- Colour reagent B: 12.5 ml of stabilized chromogen (tetramethylbenzidine).
- Stop solution: 6 ml of 2N sulfuric acid.
- Plate covers: 4 adhesive strips.

Storage:

A fresh standard was used for each assay. All other materials were stored at 2-8^0 C for up to 1 month.

Preparation of reagents:

All reagents were brought to room temperature before use.

1) Wash buffer:

If crystals formed in the concentrate, it was warmed to room temperature and mixed gently until the crystals dissolved. 20 ml of the wash buffer concentrate was diluted in deionised or distilled water to prepare 500 ml of

wash buffer.

2) Substrate solution:

Colour reagents A and B were mixed together in equal volumes within 15 minutes of use. 200 µl of the resultant mixture was required per well.

3) VEGF standard:

The VEGF standard was reconstituted with 1 ml of calibrator diluent. This stock solution produced a stock solution of 2000 pg/ml. The standard was left undisturbed for 15 minutes with gentle mixing prior to making dilutions.

4) Cell culture supernate samples: Polypropylene tubes were used. 500 µl of calibrator diluent RD5K was pipetted in each tube. The stock solution was used to produce a dilution series (Fig 11.2.4). Each tube was mixed thoroughly before transfer. The 1000 pg/ml dilution served as the high standard. The calibrator diluent served as the zero standard (0 pg/ml).

<u>Assay procedure:</u>

All reagents and samples were brought to room temperature before use and all samples were assayed in duplicate.

1) All reagents and standards were prepared.

Excess microplate strips were removed from the plate frame and returned to the foil pouch containing the desiccant pack and resealed.

3. For culture supernate samples, 50 µl of assay diluent RD1W was added to each well.

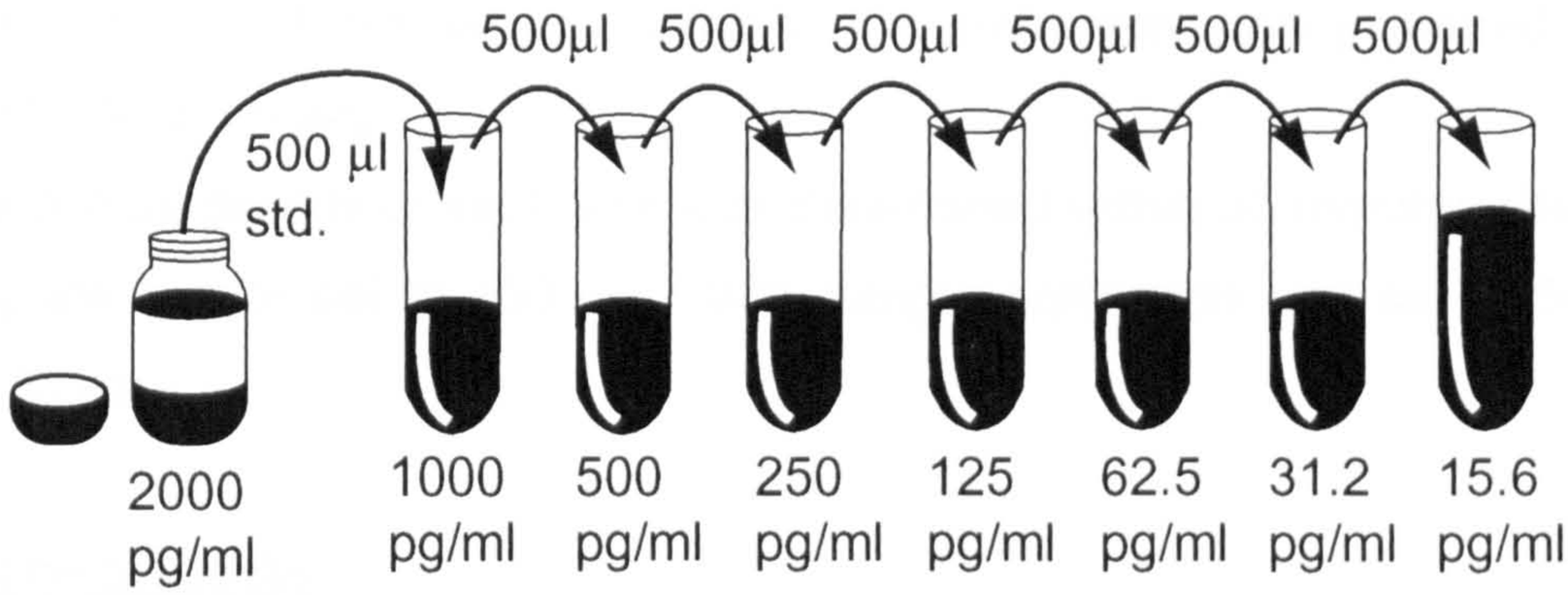
4. Then 200 µl of standard or sample was added to the well. The plate was then covered and incubated at room temperature for 2 hours.

5. Each well was then aspirated and washed with an automated washer, using 400 µl of wash buffer, at least 3 times. Complete removal of liquid at each step was mandatory. Any remaining wash buffer was removed by gently tapping on blotting paper.

6. 200 µl of the conjugate was then added into each well, the plate resealed and incubated at room temperature for 2 hours.

7. Washing was repeated, as in step 5.

8. 200 µl of the substrate solution was then added to each well and incubated at room temperature for 2 hours. The colour changed from colourless to gradations of blue.



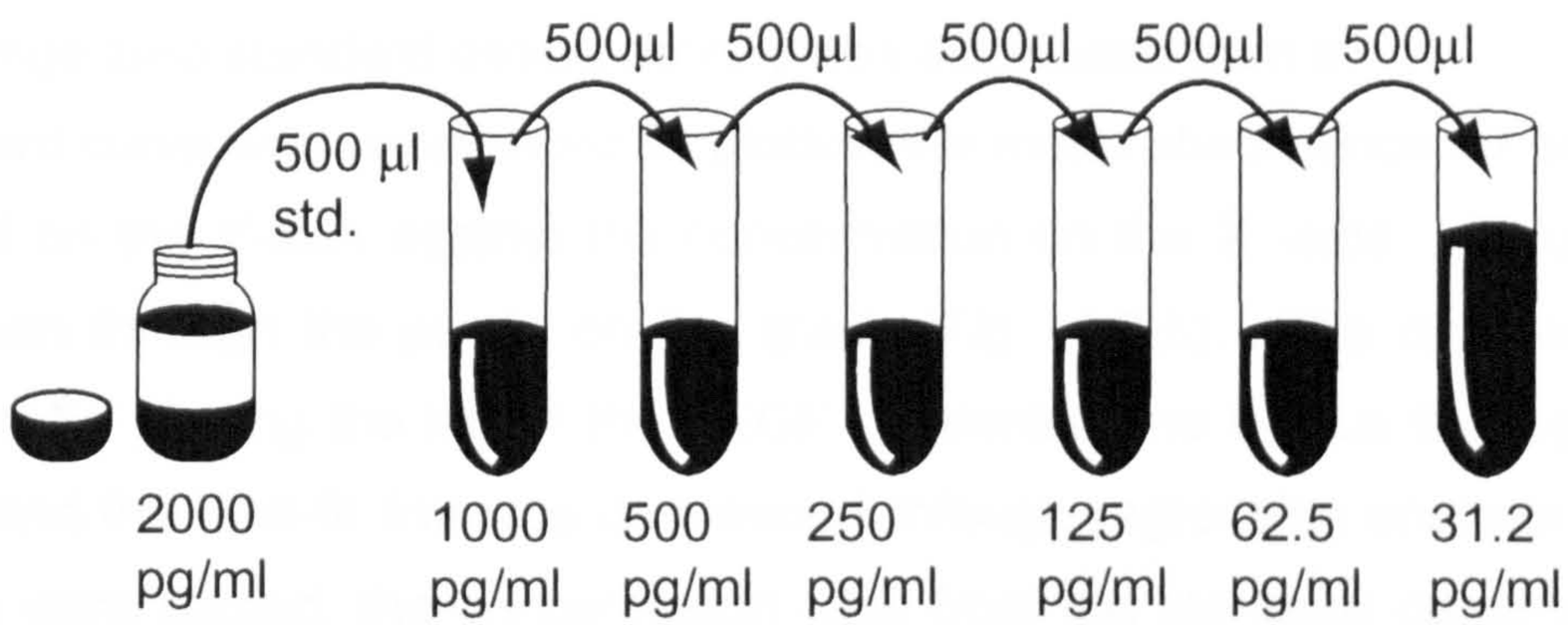
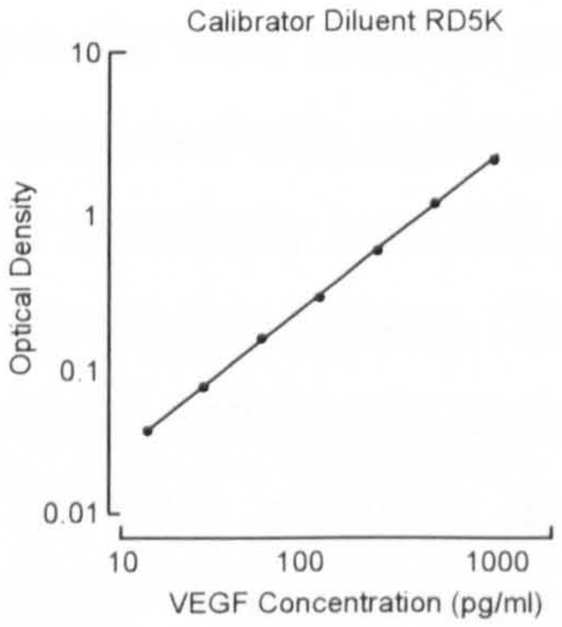
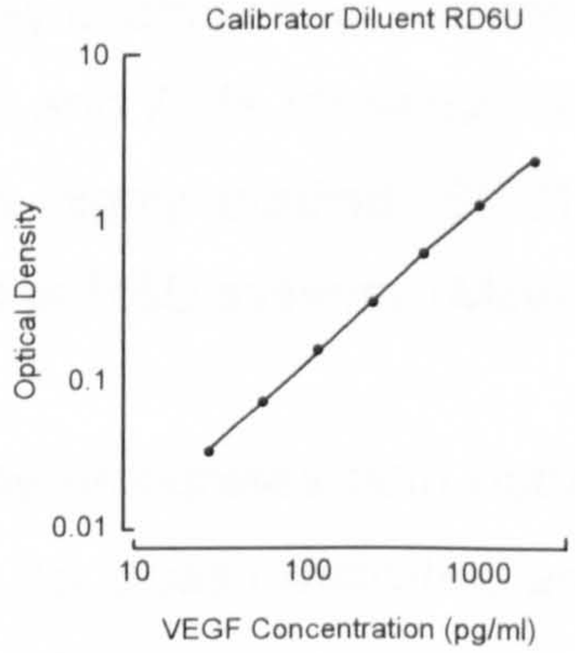


Figure 11.2.4

Serial dilutions of culture media and serum/plasma for measurement of VEGF concentrations by Quantakine enzyme immunoassay.



(pg/ml)	O.D.	Average	Corrected
0	0.074 0.076	0.075	
15.6	0.118 0.121	0.120	0.045
31.2	0.159 0.159	0.159	0.084
62.5	0.246 0.242	0.244	0.169
125	0.384 0.378	0.381	0.306
250	0.666 0.669	0.668	0.593
500	1.258 1.263	1.260	1.185
1000	2.302 2.233	2.268	2.193



(pg/ml)	O.D.	Average	Corrected
0	0.068 0.071	0.070	
31.2	0.107 0.110	0.108	0.038
62.5	0.149 0.153	0.151	0.081
125	0.230 0.230	0.230	0.160
250	0.377 0.387	0.382	0.312
500	0.657 0.699	0.678	0.608
1000	1.261 1.281	1.271	1.201
2000	2.159 2.246	2.202	2.132

Figure 11.2.5

Standard curves for VEGF using Quantakine enzyme immunoassay. (Figures 11.2.4 and 11.2.5 reproduced from information leaflets supplied in the Quantakine immunoassay kit).

9. 500 µl of stop solution was then added in each well. If the colour change (blue to yellow) did not appear uniform, the plate was gently tapped to ensure thorough mixing.
10. The optical density of each well was determined within 30 minutes using a microplate reader set to 450 nm. Wavelength correction was set to 540 nm or 570 nm.

Calculation of results:

Duplicate results for each standard, control and sample were averaged and the average zero standard optical density was subtracted from it.
A standard curve was constructed by plotting the mean absorbance for each standard on the Y-axis against the concentration on the X -axis. A curve was drawn through the points on the graph (Fig 11.2.5). The data were linearised by plotting the log of the VEGF concentrations versus the log of the OD and the best-fit line was determined through regression analysis. If samples were diluted, the concentration read from the standard curve was multiplied by the dilution factor.

Each kit had enough reagents for two 96-well immunoassay plates. Duplicate wells were read for sample and standards. A total of 82 samples could be analysed with each kit in duplicate. The assay time was 4.5 hours. The assay sensitivity was 5.0 pg/ml for cell culture supernatants and 9.0 pg/ml for serum and plasma. The minimum detectable amount of VEGF was determined by adding two standard deviations to the mean optical density value of 20 zero standard replicates and calculating the corresponding concentration. The intra-assay coefficient of variation was 4.7% and the inter-assay coefficient of variation was 6.7% for cell culture supernate assay and 5.4% and 7.3% for serum respectively. The immunoassay is calibrated against a highly purified, *Sf* 21 expressed, recombinant human $VEGF_{165}$ produced at R&D systems (Minneapolis, MN, USA).

The assay recognises both natural and recombinant $VEGF_{165}$. There were however, no cross reactivities against WHO cytokine standards: (Human IL-1alpha, IL-1beta, all other types of IL-1 to IL-13, ANG, AR, CNTF, B ECGF,

EGF, Epo, aFGF, bFGF, FGF-4, 5, 6 AND 7, TNF alpha, GM-CSF, G-CSF, M-CSF, SGP130, GRO alpha, GRO beta, GRO gamma, HB-EGF, HGF, IGF-1, 2, LAP, LIF, MCP, all types of MIP, NGF, OSM, PD-ECGF, PDGF-AA, AB, BB, PTN, RANTES, SCF, SLP1, and all types of TGF and TNF. The $VEGF_{165}$/PlGF heterodimer exhibited approximately 20% cross-reactivity over the range of immunoassay.

The assay was validated by parallelism and quantitative recovery. To assess linearity of the assay, five samples were spiked with high concentrations of VEGF in various matrices and diluted with appropriate calibrator diluent to produce samples with values within the dynamic range of the assay (Table 11.2). The kit was stored until used, at 4^0 C and the assay performed at room temperature.

Recovery: The recovery of VEGF spiked to three different levels in five samples throughout the range of the assay are shown in Table 11.2.

Table 11.2: The recovery of VEGF spiked to three different levels in five samples throughout the range of the assay.

Sample type	Average % recovery	Range
Cell culture media	102	95-115%
Serum	102	92-115%
EDTA plasma	97	82-113%
Heparin plasma	93	82-102%
Citrate plasma	100	88-113%

Expected values: 37 matched human serum, EDTA plasma, heparin plasma and citrate plasma samples were assayed. The results are shown in table 11.3.

Table 11.3: The mean detectable VEGF in human serum, EDTA plasma, heparin plasma and citrate plasma samples (n = 37).

Sample type	% detectable	Mean detectable VEGF (pg/ml)	Range (pg/ml)
Serum	100	220	62-707
EDTA plasma	24	61	ND-115
Heparin plasma	22	41	ND-55
Citrate plasma	0	-	ND

The results of this experiment show that serum is the most optimum sample for measuring VEGF concentrations. Measuring VEGF concentrations in plasma samples may not result in detectable VEGF concentrations.

Cell culture supernates:

Human peripheral blood mononuclear cells (10,0000 cells/well) from healthy normal donors were cultured and observed for 5 days. The cells were cultured unstimulated or stimulated with 10 μg/ml PHA for 1 to 5 days. The recovery results are shown in table 11.4.

Table 11. 4: VEGF recovery from cell cultures unstimulated or stimulated with PHA.

Activation Condition	Day 1 (pg/ml)	Day 5 (pg/ml)
Unstimulated	356	332
Stimulated	14	1440

The results of this experiment show the percentage recovery of VEGF in cell culture supernates obtained from mononuclear cells stimulated with PHA was higher compared with those obtained from cells which were unstimulated with PHA. VEGF concentrations declined steadily in cells, which were not stimulated with PHA.

Assay linearity between the two assay techniques:

In order to assess the assay linearity of the two assay systems, the regression curve and the Pearson's correlation coefficient between VEGF measurements obtained by Cytokit Red™ immunoassay and the Quantakine immunoassay system were determined. The two assays were performed by Nadia Payne. The results of these are shown in (Fig 11.2.6). The absolute explored values were 10 times higher with Cytokit enzyme immunoassay after swapping standards. The internal calibration of each assay was different and we had no independent source of VEGF to test.

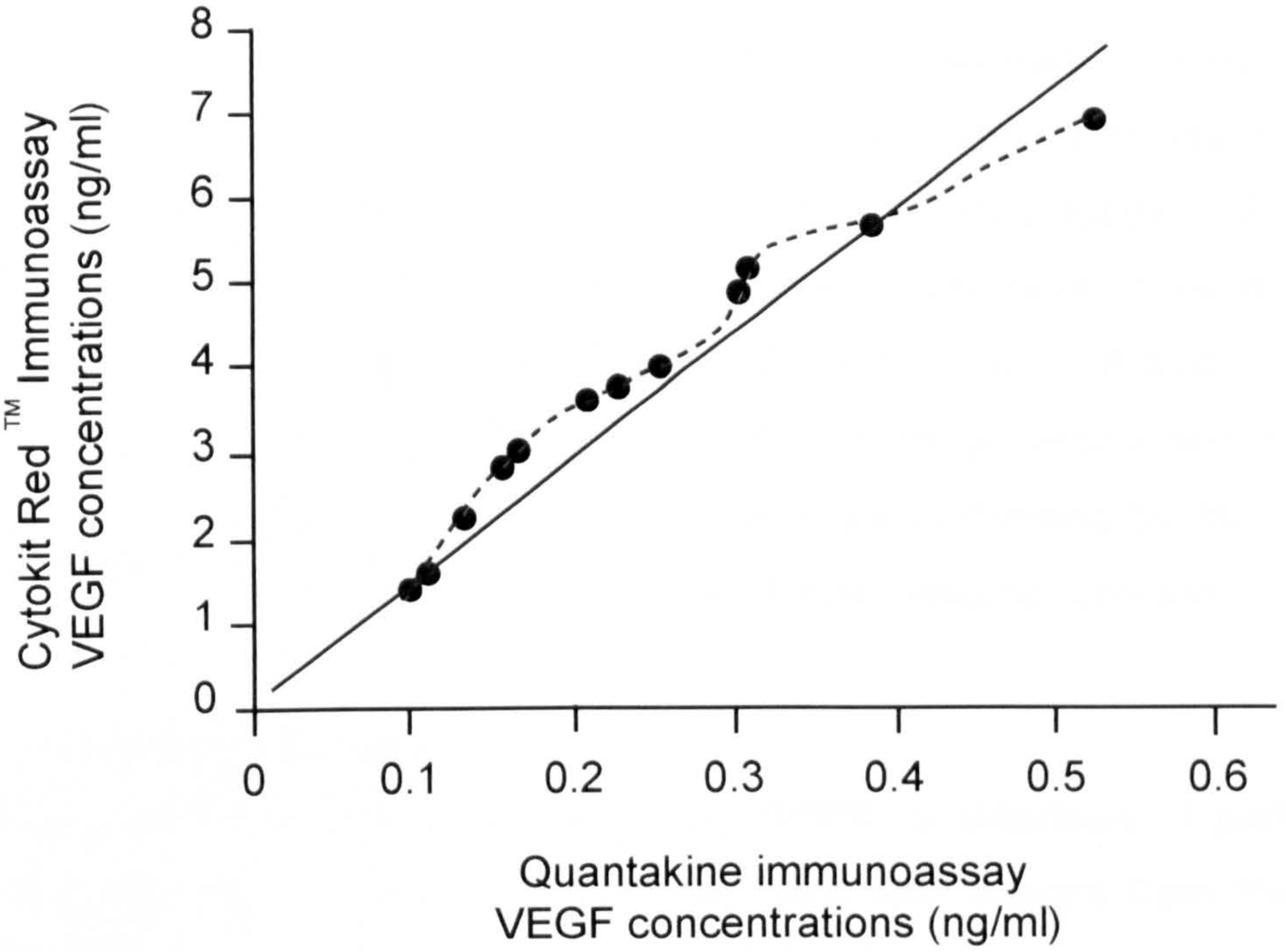


Figure 11.2.6:

Correlation coefficient assay linearity and regression curve as determined by Cytokine and Quantakine assays for measurement of VEGF concentrations in serum. The blue dotted represents the 'best fit' curve and the red line the reference line.

II v) Hormone assays:

FSH and LH were measured by microparticle enzyme immunoassay (Abbot AxSYM reagent pack, Chicago, Illinois, USA) and oestradiol, progesterone (both in serum and cell culture media) and testosterone by radioimmunoassay (Sorin Clinical Assays and Coated tubes, DPC, Los Angeles, CA, USA). The intra and inter-assay coefficients of variation (as calculated by Nadia Payne) were 2.9 and 3.7 % for FSH, 1.8 and 4.1 % for LH, 6 and 7.5 % for oestradiol, 6.2 and 8% for progesterone and 6.6 and 9.2% for testosterone. All hormone assays were performed by Mrs. Nadia Payne at the Cobbold Laboratory, The Middlesex Hospital, London.

II vi) Statistical analysis:

Statistical analyses were performed using SPSS for Windows. I performed all statistical tests. Advice for statistical tests was sought from Prof. HS Jacobs and Dr. GS Conway. Dr. Sarah Wild, Lecturer in Epidemiology at The London School of Tropical Medicine and Hygiene at London, performed statistical tests in study 4. The details of this are given below.

Data are represented as mean $\pm$ SD in all studies. *P* values of < 0.05 were considered significant. Student's t - test was used to compare continuous variables. Correlations between variables were sought by Pearson's correlation coefficient in all studies. Hormone and VEGF concentrations were log transformed for parametric analysis.

For study 1, comparisons between the three phases of the menstrual cycle were performed by analysis of variance (ANOVA). Post hoc analysis was undertaken using Scheffé's test. Power calculation of the study was performed after analysis of results from 6 women. The analysis showed that 11 patients were necessary to show 90% power detection. Covariate analysis was performed using general factorial ANOVA. Comparisons between VEGF concentrations between women and men were made using the independent t- test.

In study 2, comparisons between the three groups of women were

performed by analysis of variance (ANOVA).

For study 3, comparisons between the three groups were performed by analysis of variance (ANOVA) using post-hoc Scheffés test. For the analysis of results in women with PCO and with normal ovaries on ultrasound, age of the women was used as a covariate because of the higher mean age of the women with normal ovaries compared with that in women with PCO and PCOS (Table 1).

For study 4, hormone concentrations were logarithmically transformed to normalise the distribution of data. Mean values were compared across the categories of OHSS by analysis of variance. Post - hoc analysis was performed using Scheffés test. A likelihood ratio test, based on logistic regression modelling using unit change in each predictive criterion of OHSS, was used to test whether VEGF concentrations made a significant contribution to the prediction of OHSS. The sensitivity, specificity and positive predictive value of various markers for OHSS in this study were investigated and compared. The sensitivity of a test (true positive rate) is defined as the ratio of true positive tests divided by the sum of true positive and false negative tests. The specificity of a test (true negative rate) is defined as the ratio of the true negative tests divided by the sum of false positive and the true negative tests. The positive predictive value (post - test probability of a positive test) is defined as the ratio of true positive tests divided by the sum of true positive and false positive tests. The optimum cut - off values for risk prediction for various variables were identified from receiver operating curves (ROC).

For study 5, comparisons between the different treatment groups were performed by analysis of variance (ANOVA). Post hoc analysis was undertaken using Scheffé's test.

For study 6, data were not normally distributed and were therefore analysed using non-parametric analyses. Comparisons between VEGF concentrations pre and post-hysterectomy were performed using Wilcoxon

signed rank test. The Mann - Whitney U test, which compares 2 independent continuous variables, was performed to analyse differences in VEGF concentrations between the two groups of women prior to and after hysterectomy.

In study 7, data were not normally distributed and therefore analysed using non-parametric analysis. Mann - Whitney U test was used to compare 2 independent continuous variables (log values) and the Kruskall - Wallis test was used to measure multiple variables.

To test the assay linearity between the two assay techniques (Cytokit Red™ and Quantakine immunoassays), coefficient correlation test and regression curve analyses were performed.

Section III) Materials and Methods for laboratory studies:

Clinical Materials: Human granulosa cells were obtained from women undergoing ovarian stimulation cycles as a part of in-vitro fertilisation treatment at The London Women's Clinic. The granulosa cells were isolated after the patients had been given hCG injection and were therefore, in the final stages of granulosa cell differentiation, i.e. granulosa lutein cells. Follicular fluid obtained during oocyte retrieval was used for the in-vitro culture studies, which were performed the same day.

I performed all in-vitro culture experiments at The Cobbold Laboratories, The Middlesex Hospital, London. Dr. Debbie Willis, who is a Post Doctoral Fellow at The St Mary's Hospital, London, taught me the technique of in-vitro culture of granulosa cells.

Cell culture materials:

Cell culture reagents were obtained from Life Technologies Ltd., Paisley, Scotland, unless otherwise stated.

1) Culture medium was Medium 199 (M199) with 25 mM HEPES, Earle's salts and L-glutamine. M199 was supplemented with additional L-glutamine to give a final concentration of 2.8 mM L-glutamine, penicillin and streptomycin (50 IU/ml) and 1% fetal calf serum before use.
2) Dulbecco's Phosphate buffered saline (PBS) without $NaHCO_3$.
3) Hanks Balanced Salt solution (HBSS).
4) $NaHCO_3$ (7.5%).
5) Percoll (Pharmacia, St. Albans, Herts, UK).
6) Trypan blue stain (0.4% in 0.81% sodium chloride and 0.06% potassium phosphate) from Sigma Chemical company, Poole, Dorset, UK.
7) 1% Fetal calf serum.
8) Testosterone (Sigma Chemical company).
9) Gonadotrophin preparations were highly purified human pituitary luteinising hormone (activity 12700 IU/mg; FSH 7.0 IU/mg, LHadi, Serono Pharmaceuticals, Welwyn Garden City, Herts, UK), follicle stimulating hormone (activity 15,000 IU/mg; LH 16 IU/mg, Gonal-F, Serono

Pharmaceuticals) and human recombinant hCG (Serono Pharmaceuticals, Welwyn Garden City, Herts, UK).

10) Human recombinant Insulin (25.7 IU/mg) was obtained from Boehringer Mannheim Biochemica, Lewes, East Sussex.

Validation tests:

In 1997, Dr. Debbie Willis during in-vitro culture on granulosa cells, conducted validation studies to assess the effects of different culture conditions on granulosa cell function. The results of these validation studies on granulosa cell culture formed the basis of the principles adopted in the studies described below, for choosing plating density of the cells and the concentration of testosterone. The validity studies also demonstrated the ability of granulosa cells to respond to LH and assessed the purity of granulosa cells. Since the methodologies of the experiments have been adopted from the same Department as Willis, these experiments were not repeated. The results of the experiments as performed by Dr. Debbie Willis are briefly described. These have been described in her thesis (Willis D, 1997)

1) Plating density: Granulosa cells were cultured at a range of plating densities (12.5 - 400 x 10^3 cells/well) in 500 µl Medium 199 for 48 hours. Progesterone accumulation in the granulosa cell conditioned medium was determined by radioimmunoassay. Increasing plating density was found to increase progesterone accumulation/1000 cells and hence per cell.

A plating density of 50,000 cells / well was chosen because there was a relatively small variation in progesterone accumulation /100 cells around this density and hence small variations in plating density would have small effects on progesterone accumulation.

2) Testosterone dose response:

A testosterone dose response curve was established to determine the optimum concentration of this substrate for aromatase in the granulosa cell

cultures. Granulosa cells at 50,000 cells / well were incubated with a range of doses of testosterone ($5x10^{-8}$ M - $5x10^{-5}$ M) with or without LH, for 48 hours. The effect of testosterone on E_2 and progesterone accumulation was observed. It was observed that incubation of granulosa cells with testosterone enhanced E_2 production in a dose dependent manner. A plateau of E_2 production was consistently reached at a concentration of $5x 10^{-7}$ M. There was no effect of testosterone at the lower doses ($5x10^{-8}$ M - $5x10^{-7}$ M) on progesterone accumulation. Higher concentrations of testosterone however, suppressed progesterone accumulation. The presence or absence of LH did not affect the concentration of testosterone at which this plateau was reached.

On the basis of these findings a testosterone concentration of $5 x 10^{-7}$ M was chosen for the following experiments as it gave maximal E_2 production whilst having no effect on progesterone accumulation.

3) <u>Ability of granulosa lutein cells to respond to LH:</u>

As a part of the protocol for IVF treatment, patients received an injection of hCG, 36 hours prior to oocyte retrieval. Since hCG binds to the LH receptor, it was important to establish whether administration of hCG in vivo interfered with granulosa cell response to LH in-vitro. The aim was to observe response of these cells, if challenged by insulin, LH and LH + Insulin, in the presence of testosterone.

It was observed that LH increased progesterone production after a pre incubation period of 48 hours. Basal progesterone was maximal 48 hours after culture. Insulin had no effect on progesterone or E_2 production but addition of LH to insulin augmented progesterone production.

4) <u>To assess the purity of granulosa cell culture:</u>

Granulosa cell cultures were tested for contamination with thecal cells by measuring androstenedione levels in granulosa cell conditioned medium in the presence of LH. In contrast to theca cells, granulosa cells do not contain the enzyme cytochrome P450 17 alpha, 17-20 lyase, which is essential for androstenedione synthesis. Hence addition of LH should not affect

androstenedione concentrations in granulosa cell culture media.
There was no effect of LH on the production of androstenedione in granulosa cell cultures although it augmented progesterone production in a dose dependent manner.

These culture conditions, as established by Dr. Debbie Willis were maintained for all in-vitro culture studies on granulosa cells conducted thereafter.

Patients:

Human granulosa cells were isolated from pooled follicular aspirates obtained during vaginal oocyte retrieval from 20 women undergoing stimulation cycles as a part of in-vitro fertilisation (IVF) treatment. Ten women had normal ovaries and ten had PCO/PCOS as defined by the criteria of Adams et al., 1995. The granulosa cells were isolated from follicular fluid after the patients had been given hCG and were therefore in the final stages of granulosa cell differentiation, i.e. granulosa lutein cells. Patients recruited for the study underwent the long-term pituitary suppression protocol for IVF, which is described earlier (page 105). Ovarian stimulation was achieved using HMG (Menogon, Ferring Pharmaceuticals, Langley, Berkshire, UK). Follicular fluid obtained during oocyte retrieval was used for the in-vitro culture studies, which were all performed on the day of collection of follicular fluid.

In order to optimise culture conditions a time and a dose response curve was performed on 4 women initially, two of whom had normal ovaries and two had PCO/PCOS. (Table 11.5, Patients 1 - 4). Table 11.5 describes the characteristics of these women.

We subsequently recruited 8 further women with normal ovaries and 8 with PCO/PCOS. Comparison of VEGF release in cell culture media from granulosa lutein cells was therefore made from a total of 10 women with normal ovaries and 10 with PCO/PCOS.

There were no differences between the age, BMI, duration of infertility, menstrual cycle pattern, parity, treatment diagnosis, duration of ovarian stimulation, dose of gonadotrophins used to achieve ovarian stimulation, baseline FSH, LH and testosterone concentrations and the number of patients achieving pregnancy between women who had normal ovaries and those who had polycystic ovaries/PCOS. Statistically significant differences were however, observed between the number of follicles recruited, oocytes retrieved and the number of patients developing OHSS (Table 11.6) between women with normal ovaries and PCO/PCOS.

Four women with PCO/PCOS (n = 10) developed OHSS (2 had mild and 2 had moderate OHSS as defined by the criteria of Golan et al., 1989). Women with normal ovaries (n = 10) did not develop OHSS.

Within the "PCO" group, there were no differences between the age, BMI, duration of infertility, menstrual cycle pattern, parity, treatment diagnosis, duration of ovarian stimulation, dose of gonadotrophins used to achieve ovarian stimulation, baseline FSH, LH and testosterone concentrations and the number of patients achieving pregnancy between women who developed OHSS and those who did not develop OHSS (data not shown).

<u>Isolation of granulosa lutein cells from follicular aspirate and flush:</u>

Granulosa lutein cells from pooled follicular aspirate and flushes were centrifuged at 200 g for 5 minutes, washed and resuspended in Dulbecco's phosphate buffered saline (PBS). Granulosa cells were separated from red blood cells on a 45% Percoll gradient. The cells were washed twice and resuspended in complete Medium 199 (M199) with 25 mM HEPES, Earle's salts and L-glutamine, for subsequent culture. M199 was supplemented with additional L-glutamine to give a final concentration of 2.8 mM L-glutamine, penicillin and streptomycin (50 IU/ml) and 1% fetal calf serum. Fetal calf serum was used to facilitate cells adhering to the surface of the plate. Granulosa cell number and viability were determined by Trypan blue stain exclusion test at the end of the pre-incubation period of 48 hours and at each incubation period of 1, 2, 4, 6, 9 and 12 days. The viability of

granulosa-lutein cells was typically > 85%. The final cell number did not change significantly.

Culture of granulosa lutein cells:

Granulosa cells were cultured in 24-well plates for 12 days, with medium changes every 48 hours. The cells were plated at a density of approximately 50,000 cells per well in 500 microlitre (µl) of complete Medium 199 with addition of testosterone 5 x 10^{-7} M and 1% fetal calf serum. The cell were incubated in 5% CO_2 in a humidified incubator at 37^0C. The oxygen tension (PO_2) within the incubator was kept constant and was similar to atmospheric PO_2.

After pre-incubation for 48 hours, the cells were incubated with four different concentrations of hCG (0, 0.1, 0.5, 1, and 5 IU/l), FSH (0.1, 0.5, 1, 5 iu), LH (0.1, 0.5, 1, 5 iu), insulin (0.1 -500 ng/ml) and insulin with addition of hCG for 12 days. Although physiologically FSH is not expected to alter VEGF concentrations within granulosa cells, the effect of FSH on VEGF release was studied for the sake of completion of the study. Samples for VEGF and hormone analysis were obtained after 1, 2, 4, 6, 9 and 12 days of incubation with hormones. Negative controls comprised one well with cells but without hormones (C) and a second well with cells and the addition of testosterone alone (C+T). Cells were incubated in duplicate. Gonadotrophin preparations used were highly purified human recombinant pituitary FSH, LH, hCG and human recombinant Insulin (25.7 IU/mg).

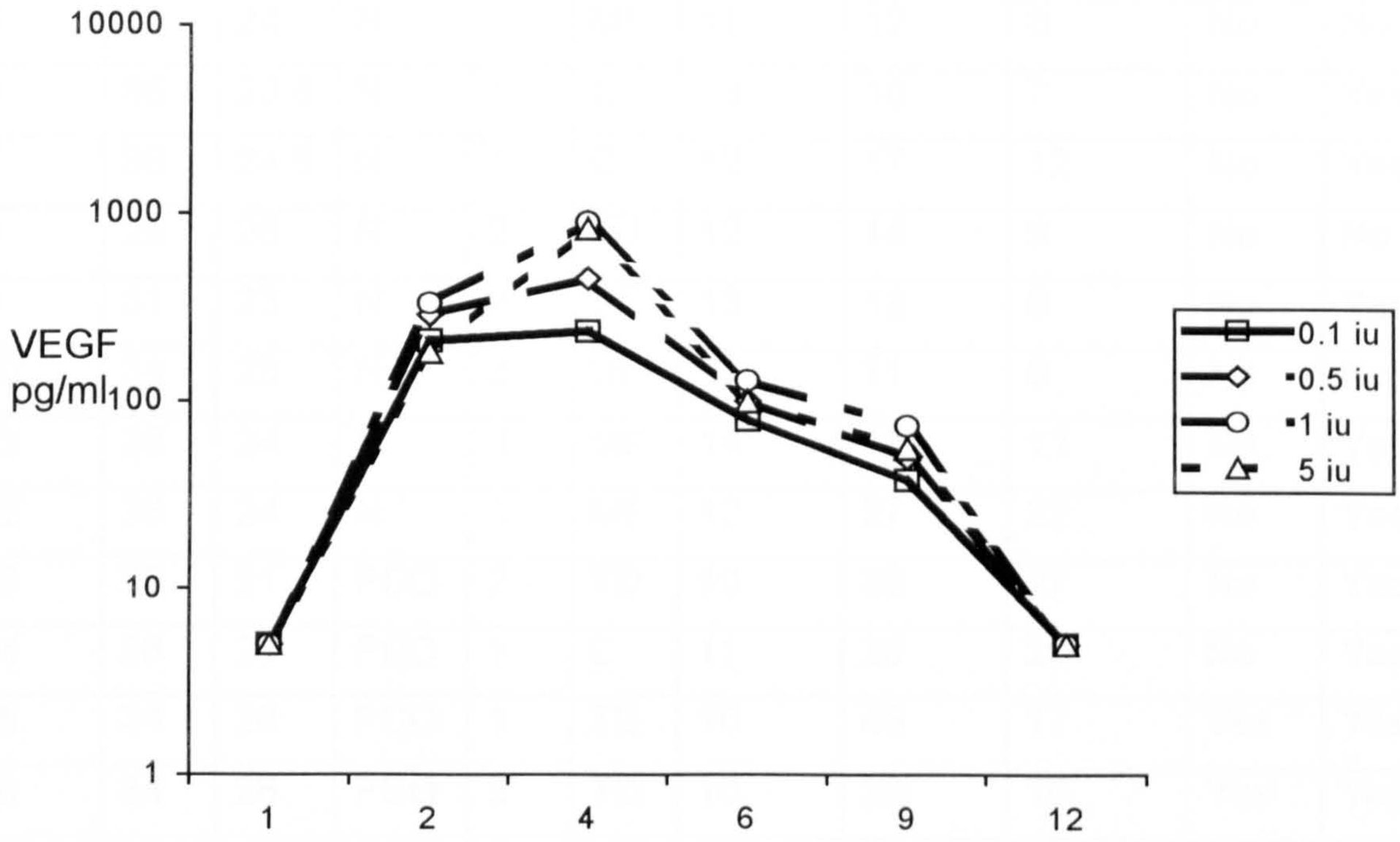
Culture samples obtained after 1, 2, 4, 6, 9 and 12 days after incubation with hormones were stored at -70 ^{0}C for analysis of VEGF, oestradiol and progesterone.

Time response curve:

Significant VEGF release by granulosa cells was observed only after 48 hours of incubation with hormones. No significant changes of VEGF concentrations were noted 24 hours after culture. Maximal VEGF release was observed after 96 hours (4 days) of culture, following which VEGF

concentrations steadily declined over 6 and 9 days of culture with almost undetectable VEGF concentrations, 12 days after culture. A time response curve was therefore established which confirmed maximum VEGF release after 96 hours (4 days) of incubation with hormones.

Fig 11.27 : Time and dose response curve: VEGF concentrations in cell culture medium, 1, 2, 4, 6, 9 and 12 days after incubation with hormone concentrations of 0.1, 0.5, 1 and 5 iu/ml of hCG (n = 4).



Dose response curve:

The effect of the hormone concentration on VEGF release by granulosa cells was observed. These consistently showed that incubation with 1 iu of each of the hormones tested resulted in maximum VEGF release compared with 0.1, 0.5 and 5 iu of hormone. A dose response curve was therefore established.

Preliminary experiment therefore, on 4 patients showed a peak response of VEGF (Fig 11.27) and progesterone production (data not shown) at 4 days of culture and at a concentration of 1 iu/ml of hormone additives (FSH, LH, hCG or hCG+Insulin. In all subsequent experiments, cells were incubated for 4 days.

Table 11.5: Characteristics of 20 patients undergoing IVF treatment who were recruited for in-vitro culture studies on granulosa lutein cells.

Patient No:	Age yrs	BMI kg/m^2	Ovary	IVF No:	Rx Diag	Days of stimulation	Follicles recruited	Oocytes retrieved	OHSS	Preg
1	28	25.6	N	2	MF	11	13	10	No	No
2	37	21	N	1	TD	10	14	12	No	No
3	39	23	PCO	1	TD	10	12	7	No	No
4	33	25	PCO	2	C	8	17	6	Yes	No
5	32	24	N	3	MF	11	12	8	No	No
6	35	23.6	N	1	C	13	10	7	No	Yes
7	36	24.5	N	1	C	12	17	12	No	Yes
8	29	26	N	2	TD	12	14	9	No	No
9	31	23	N	2	UE	13	12	9	No	Yes
10	34	28	N	3	UE	11	11	6	No	No
11	38	24	N	3	MF	14	15	11	No	Yes
12	36	24	N	1	MF	12	27	22	No	Yes
13	29	21	PCO	2	TD	10	32	27	No	Yes
14	26	28	PCO	1	C	11	35	20	No	Yes
15	34	28	PCO	1	TD	10	45	17	Yes	Yes
16	34	26	PCO	2	TD	10	33	16	Yes	Yes
17	30	19.2	BPCO	2	MF	9	20	13	No	No
18	27	19.5	PCO	3	C	11	32	26	No	No
19	29	28.7	BPCO	1	C	13	18	11	No	No
20	24	23.5	PCO	3	MF	12	12	9	Yes	No

BMI - Body mass index kg/m^2

Ovaries - N - Normal ovaries, PCO - polycystic ovaries, BPCO - borderline polycystic ovaries, which may be defined as a polycystic appearing ovary with follicle number less than 10.

IVF no: Previous IVF attempts

Rx Diagnosis - Treatment diagnosis (MF - male factor, TD - tubal disease, C - combined factors - male factor + tubal disease, UE - unexplained)

Preg - pregnancy achieved or not.

Table 11.6: Profile of women with normal ovaries and polycystic ovaries recruited in the study.

Characteristics	Normal ovaries (n = 10)	Polycystic ovaries (n = 10)	P value
Age (years)	33.4 ± 3.5	30.7 ± 4.5	NS
BMI (kg/m^2)	24.37 ± 1.9	24.19 ± 3.5	NS
No. of ampoules of gonadotrophin used	36 ± 3.4	32 ± 2.5	NS
Ovarian stimulation (days)	11.1 ± 1.5	11.2 ± 1.54	NS
Follicle number	13.2 ± 2.3	26.9 ± 4.5 *	P = 0.001
Oocytes retrieved	8.6 ± 2.2	17.2 ± 6.3 *	P = 0.001
No. of women developing OHSS	0	4 *	P = 0.001
No. of women pregnant	3	6	NS
FSH (iu/L)	6.2 ± 1.3	5.8 ± 2	NS
LH (iu/L)	4.1 ± 0.1	4.5 ± 0.9	NS
Testosterone (nmol/L)	1.2 ± 0.1	1.5 ± 0.1	NS

* Statistically significant differences

Results & Discussion:

RESULTS & DISCUSSION:

Effect of time, temperature and the sample type on VEGF concentrations measured by Cytokit immunoassay:

It was observed that serum was the optimal storage sample for measuring VEGF concentrations since heparin plasma and EDTA plasma had lower VEGF concentrations over any given storage time or temperature (Fig 12.1, 12.2, 12.3). It was observed that VEGF concentrations in heparin or EDTA plasma declined significantly after storage at any temperature over any period of time (Fig-12.1, 12.2, 12.3).

The optimum storage temperature was -70^0 C since at temperatures below this, VEGF concentrations were significantly lower (Fig12.4) than at -70^0 C.

The optimum storage time at -70^0 C for any sample type was less than 12 weeks. There were no differences in VEGF concentrations if serum was analysed for VEGF concentrations prior to this period. Analysis of serum samples after this period resulted in lower measurable VEGF concentration, which was statistically significant (Fig 12.2 and 12.3).

Discussion:

The results are consistent with results of VEGF recovery from different blood sample types as measured by Quantakine immunoassay, shown on page 128. These results may help to explain differences in VEGF concentrations reported in various studies (Krasnow et al., 1996, Abramov et al., 1997, Lee et al., 1997, Ludwig et al., 1998, Moncayo et al., 1998, Agrawal et al., 1997). All serum samples and culture medium obtained during the granulosa cell culture study were stored for less than 12 weeks at -70^0 C prior to performing VEGF assays.

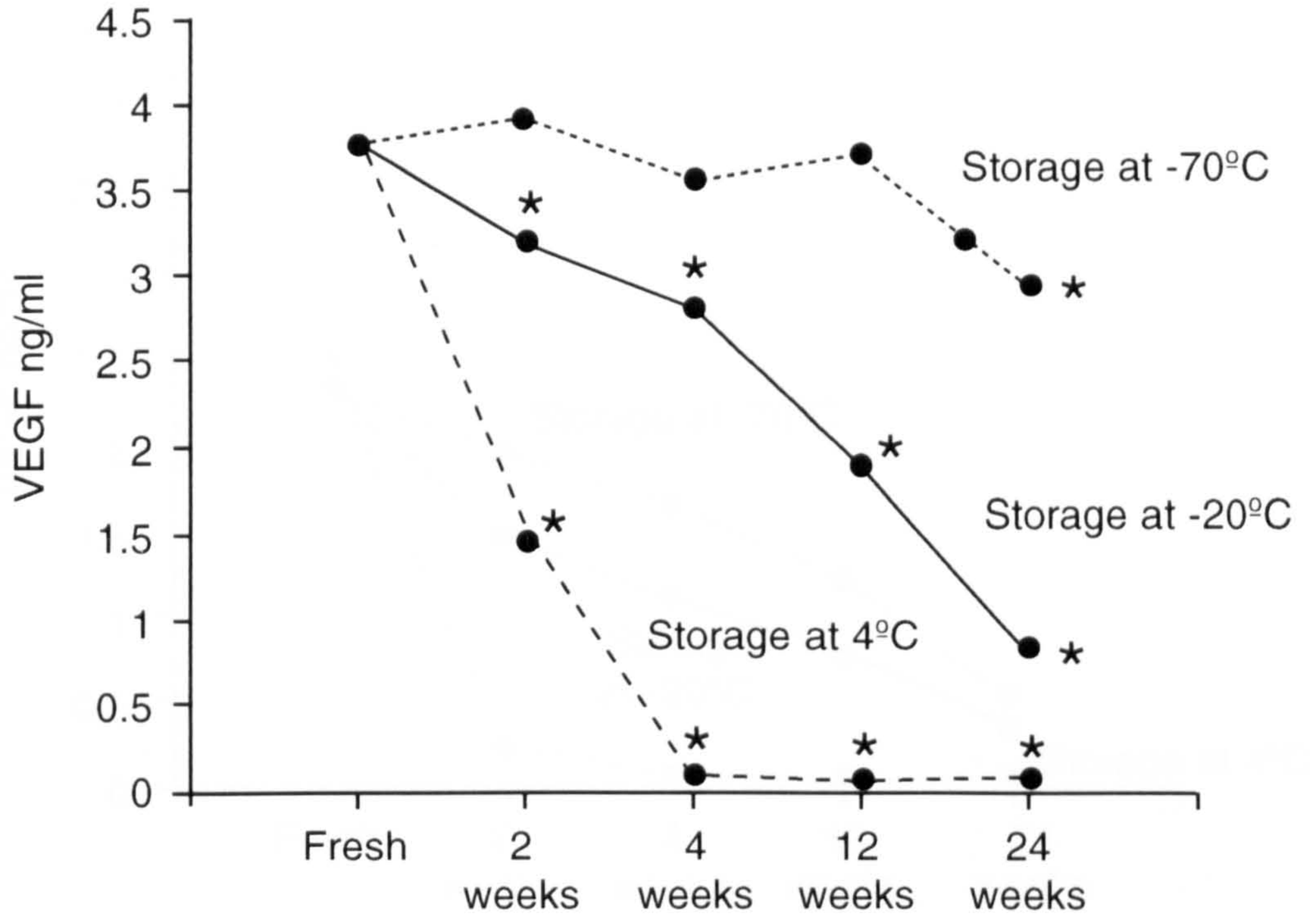


Figure 12.1:

Effect of duration and temperature of storage of serum samples on VEGF assay. Results: Mean serum VEGF at different temperatures at variable periods of time. *Significantly lower serum VEGF concentrations($p<0.001$)

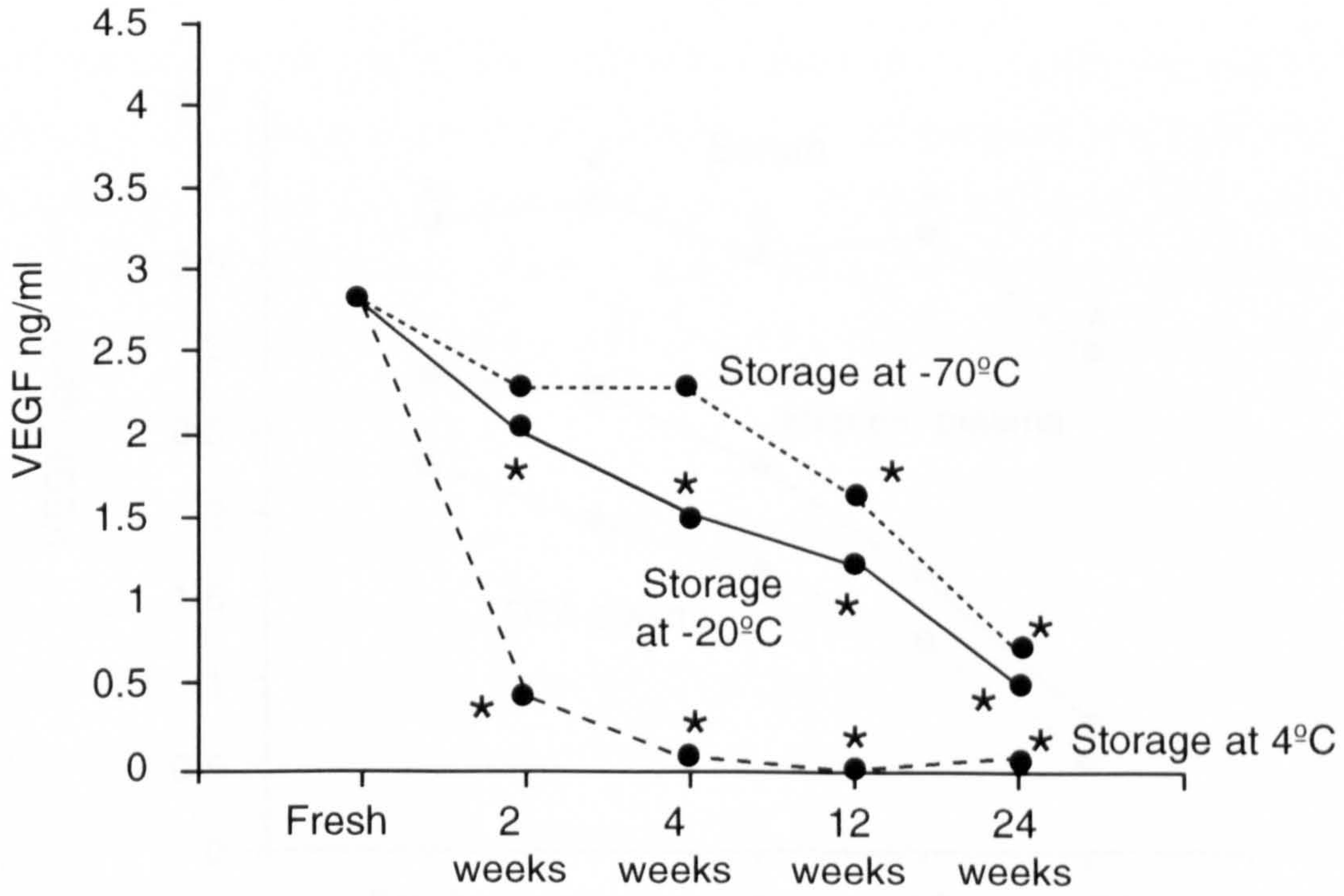


Figure 12.2:

Effect of duration and the temperature of storage of heparinised plasma samples on VEGF assay. Results: Mean heparin plasma VEGF at different temperatures at variable periods of time. *Significantly lower heparinised plasma concentrations ($p<0.001$)

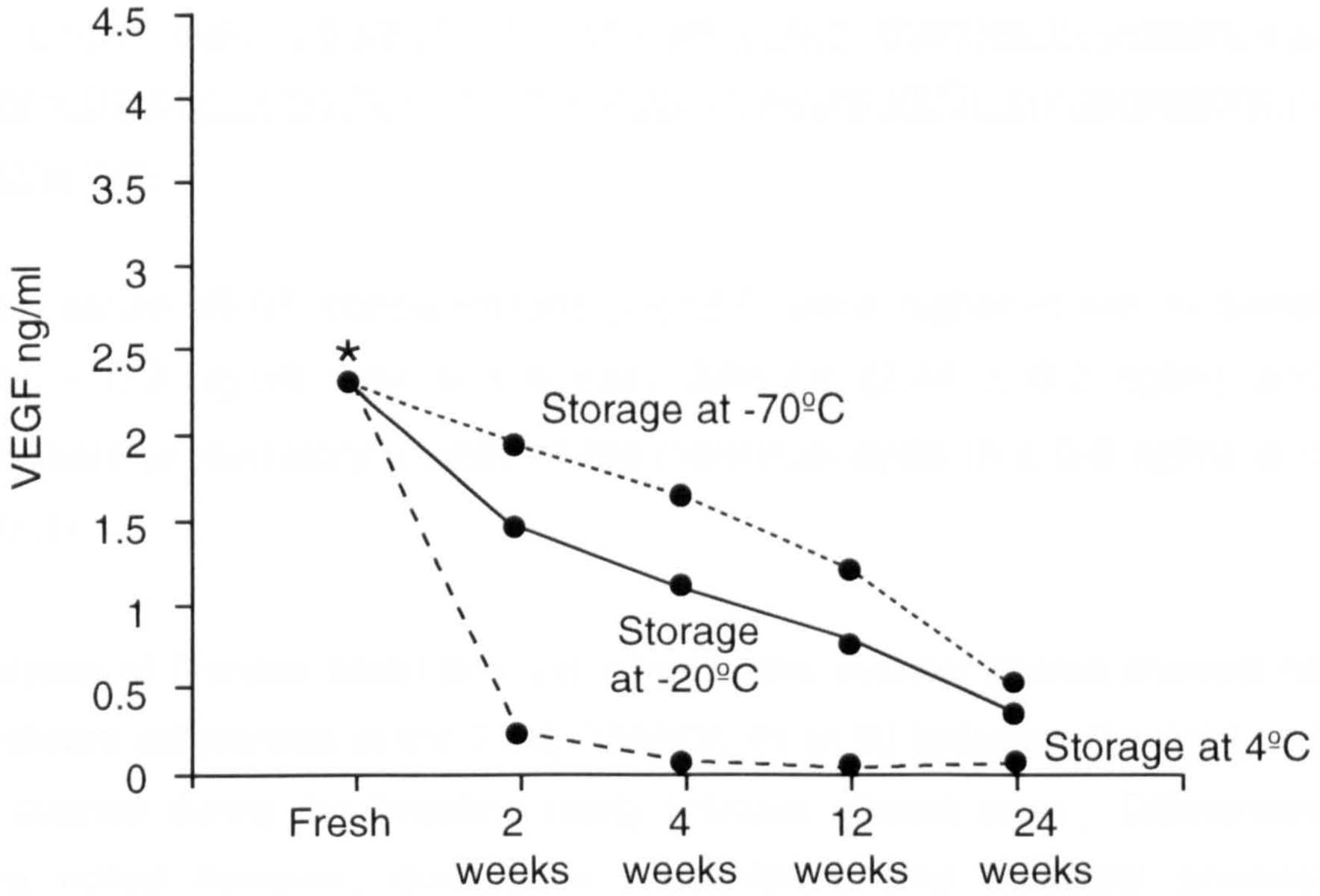


Figure 12.3:

Effect of duration and temperature of storage of EDTA plasma samples on VEGF assay. Results: Mean EDTA plasma VEGF at different temperatures at variable periods of time. All points significantly lower ($p<0.001$) than the initial point (*).

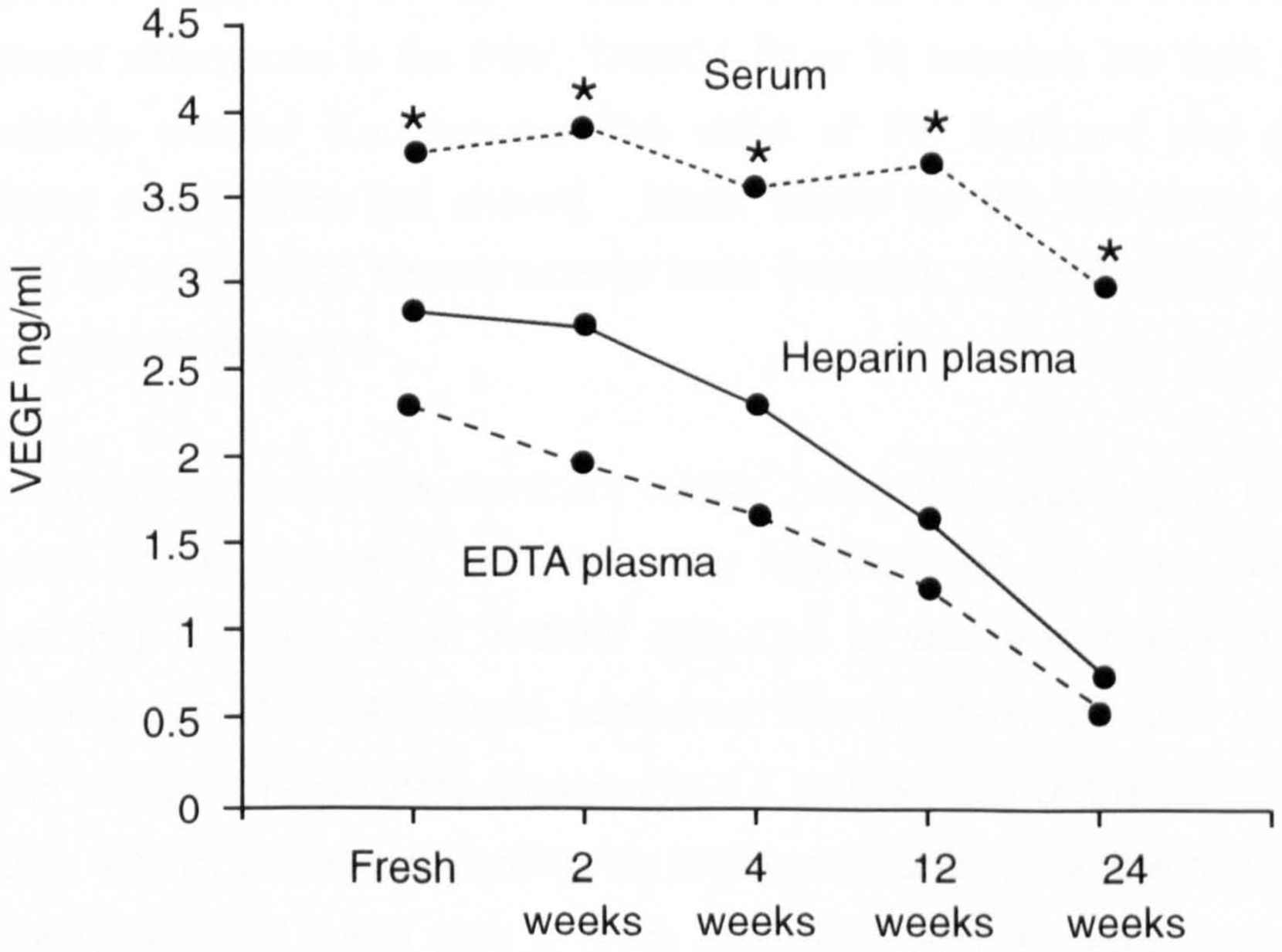


Figure 12.4:

Effect of duration of storage and blood sample type at optimal temperature (-70ºC)on VEGF concentrations. *Significantly higher VEGF concentrations ($p<0.0001$).

Study 1: The study of serum vascular endothelial growth factor (VEGF) in the normal menstrual cycle and association with changes in ovarian and uterine Doppler blood flow and the study of serum VEGF concentrations in healthy men.

Mean serum VEGF concentrations (Fig12.5) were higher in the midluteal (4.43 ± 0.9 ng/ml) than in the early follicular (2.44 ± 0.2 ng/ml) and immediate preovulatory phases of the menstrual cycle (3 ± 0.8 ng/ml, p < 0.0001).

Analyses of Doppler blood flow velocities in the ovarian stroma showed no significant differences in the PSV, TAMXV, PI or RI between the right and left ovaries during the baseline (early follicular phase) scan. Differences were noted however, during the preovulatory and midluteal phases. Subjective assessment of the ultrasound scan of the ovaries during the midluteal phase indicated a larger number of blood vessels and a greater intensity of colour blood flow within the ovarian stroma of the dominant ovary (i.e., the side of ovulation) compared with the non -dominant ovary.

Analyses of Doppler blood flow velocities of the uterine arteries showed no significant differences in the PSV, TAMXV, PI or RI between the right and left uterine arteries (i.e. between the sides of the dominant and non-dominant ovary) (data not shown). Mean values for the two blood flow velocity and resistance measurements were therefore calculated and used for subsequent analyses.

In the ovary containing the dominant follicle, mean PSV was higher in the midluteal phase compared with the early follicular and the preovulatory phases (Fig 12.6 a). Mean TAMXV (Fig 12.6 b) was also higher in the midluteal phase (17 ± 4 cm/sec) compared with the early follicular (6 ± 2 cm/sec) and the preovulatory phases (10 ± 5 cm/sec, day of LH surge, p = 0.0003). The PI decreased significantly in the preovulatory and the midluteal phases (0.78 ± 0.18 and 0.69 ± 0.14) compared with the early follicular phase (0.96 ± 0.2, p = 0.001) although no changes in RI were

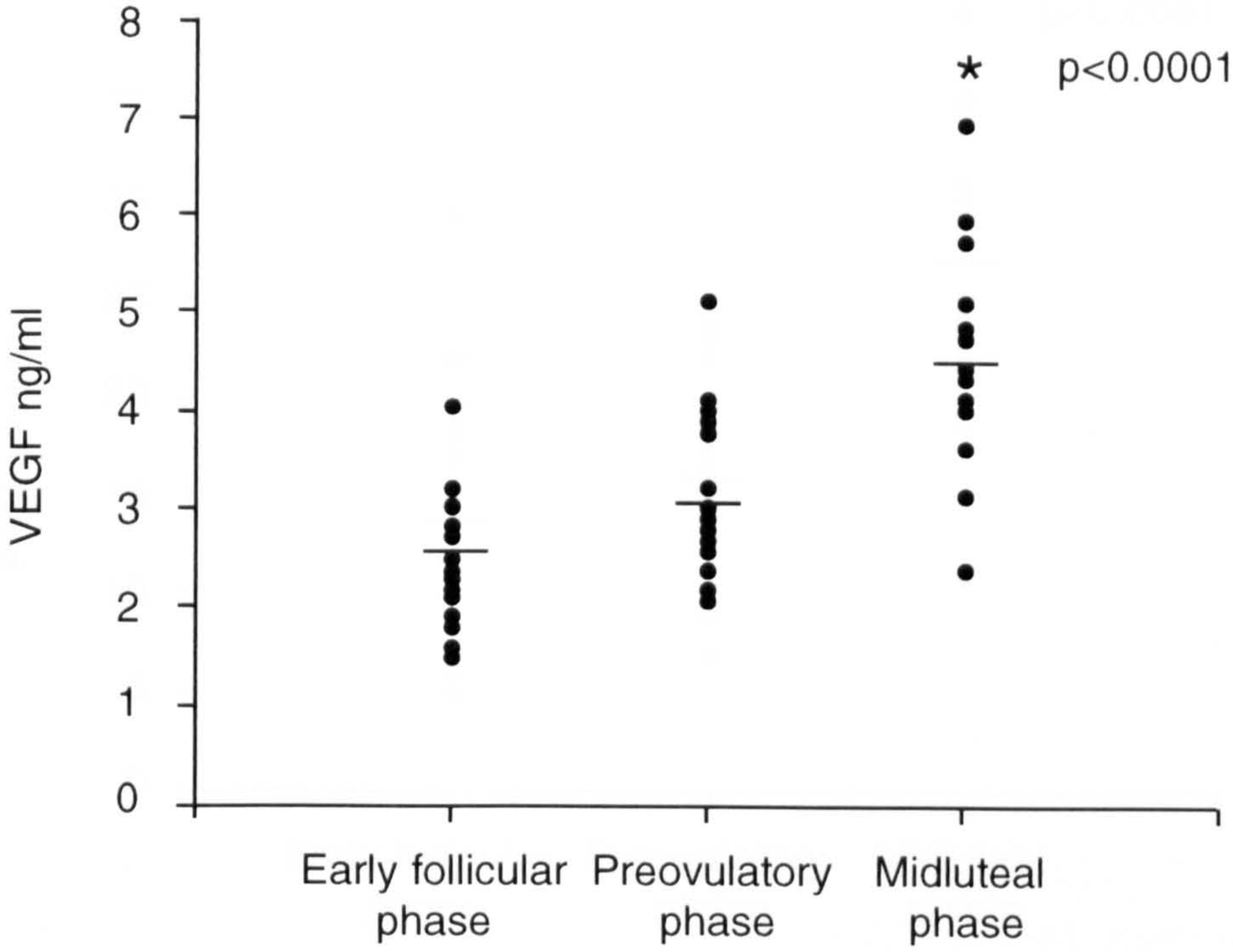


Figure 12.5:

Serum VEGF concentrations during the normal menstrual cycle.
VEGF concentrations were higher (*p<0.0001) in the mid luteal phase of menstrual cycle compared with early follicular and the preovulatory phase of the menstrual cycle.

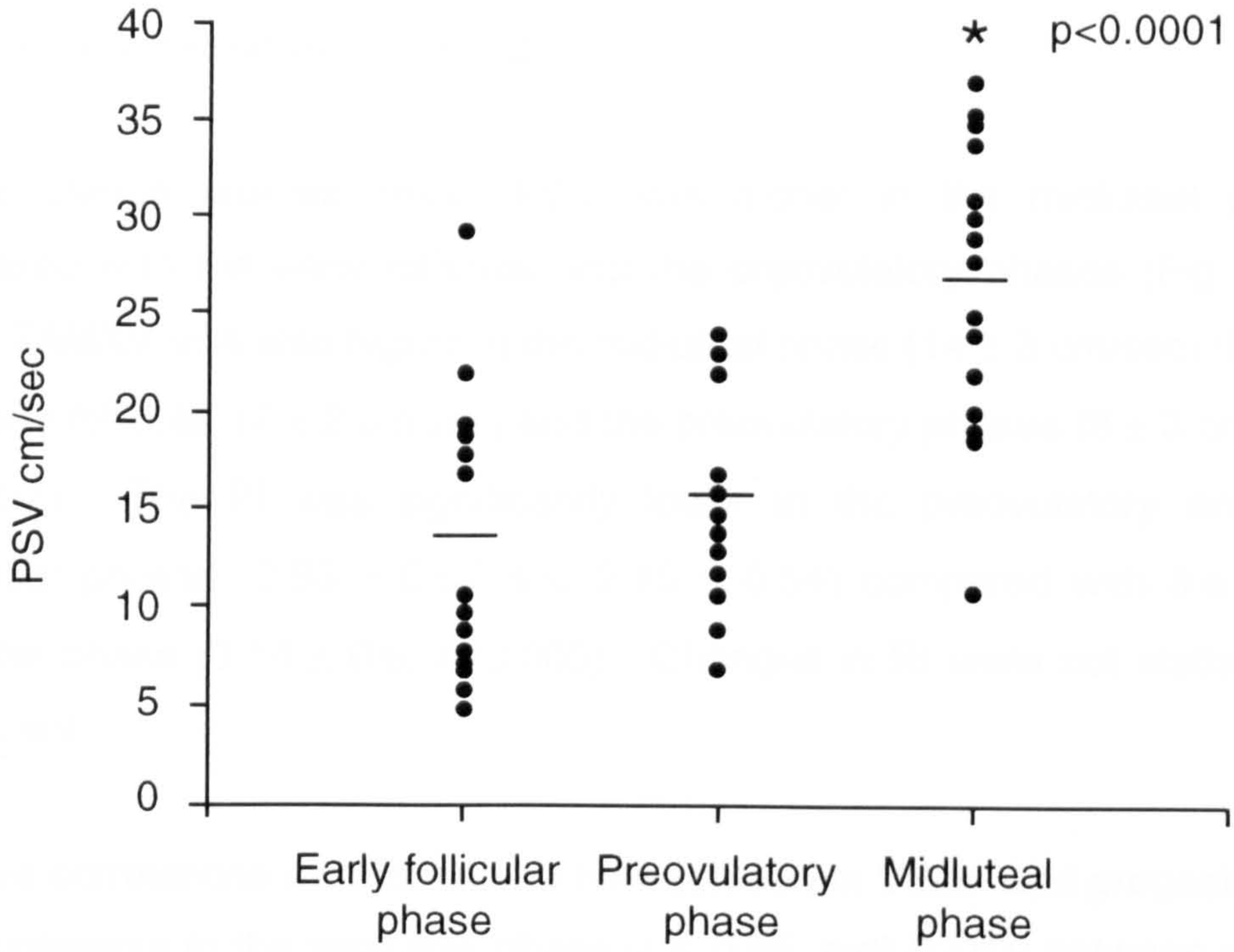


Figure 12.6(a)

Ovarian stromal peak systolic velocities (PSV) in the ovary bearing the dominant follicle, during the early follicular, preovulatory and the midluteal phases of the menstrual cycle (*p<0.0001).

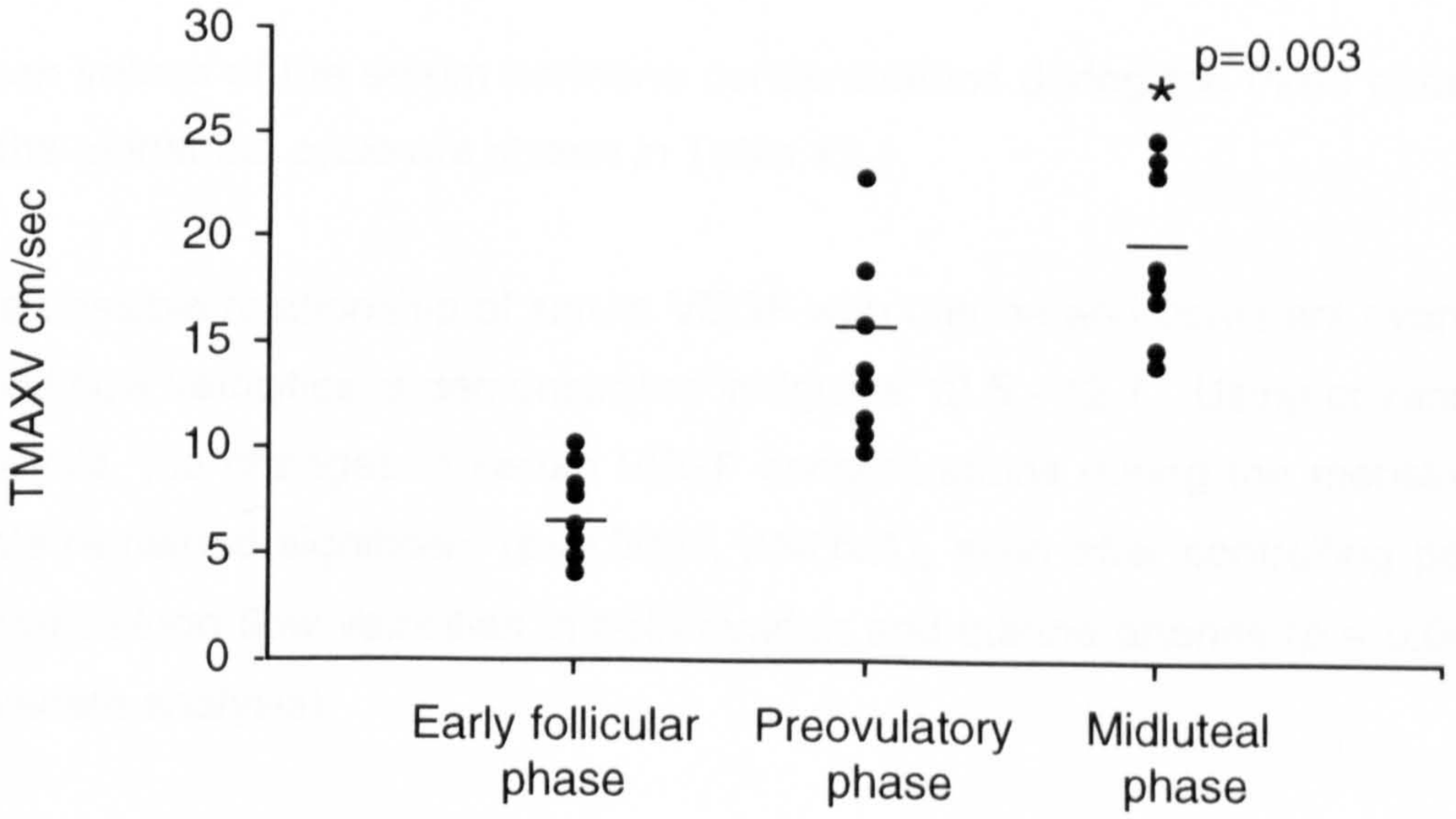


Figure 12.6(b)

Ovarian stromal time average maximum blood flow velocity (TAMXV) in the ovary bearing the dominant follicle during the early follicular, preovulatory and the midluteal phases of the menstrual cycle (*p=0.003).

detected. No cyclical changes in blood flow velocities in the non - dominant ovary were detected (data not shown).

In the uterine arteries, mean PSV was higher in the midluteal phase compared with the early follicular and the preovulatory phases (Fig 12.7). Mean TAMXV was also higher in the midluteal phase (14 ± 3 cm/sec) than in the early follicular (7 ± 2 cm/sec) and the preovulatory phases (8 ± 3 cm/sec, $p<0.007$). The PI was significantly lower in the preovulatory and the midluteal phases (2.63 ± 0.67 and 2.15 ± 0.54) compared with the early follicular phase (3.14 ± 0.6, $p<0.005$). Changes in RI were not statistically significant.

Positive correlations were observed between serum VEGF and progesterone measurements in the midluteal phase ($r = 0.85$, $p<0.0001$), between serum VEGF and oestradiol concentrations in early follicular ($r = 0.66$, $p<0.01$), preovulatory ($r = 0.57$, $p = 0.03$) and midluteal phases ($r = 0.68$, $p<0.005$) and between serum VEGF and testosterone ($r =0.69$, $p<0.01$) concentrations in the early follicular phase of the menstrual cycle. No statistically significant association of VEGF measurements with serum concentrations of LH and FSH were detected.

Mean values of the serum hormone concentrations during the three phases of the menstrual cycle are shown in Table 12.1.

The possible relationship of serum VEGF with uterine and dominant ovarian blood flow velocities is demonstrated in figures 12.5 - 12.7. Using covariate analysis, the changes in serum VEGF concentrations during the menstrual cycle remained significant ($p<0.0001$, ANOVA), even after controlling peak systolic blood flow velocities in both ovarian and uterine arteries ($p = 0.048$, covariate analysis).

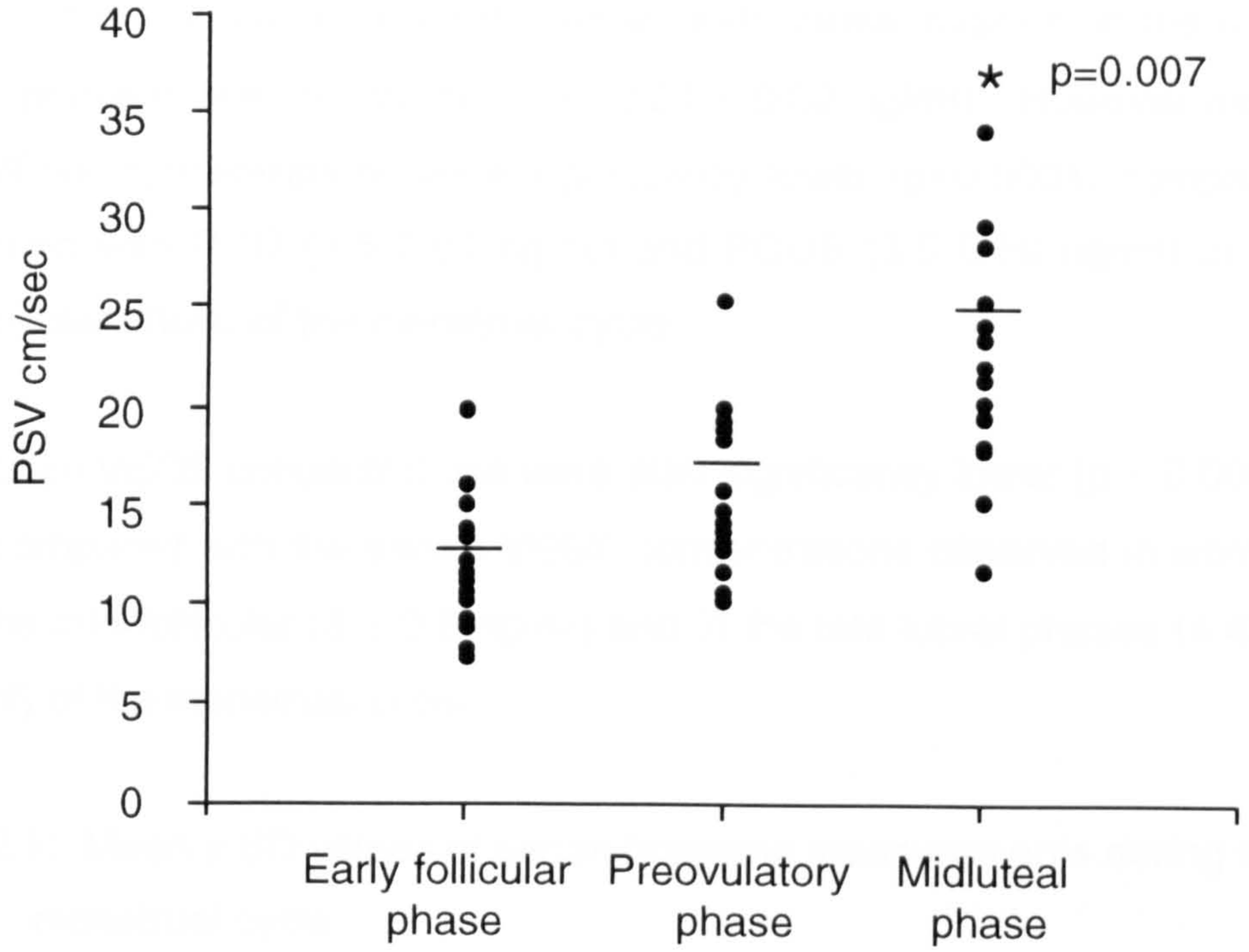


Figure 12.7:

Uterine artery peak systolic velocities (PSV) during the early follicular, preovulatory and the midluteal phases of the menstrual cycle (*p=0.007).

There were no differences in mean serum VEGF concentrations in men (2.25 ± 0.94 ng/ml) compared with women with normal ovaries, in the early follicular phase of the menstrual cycle (2.54 ± 0.62 ng/ml). However mean serum VEGF concentrations were significantly lower ($p<0.0001$) compared with women with PCO (3.5 0.61 ng/ml) and PCOS (3.5 0.59 ng/ml) in the early follicular phase of the menstrual cycle.

Mean serum VEGF concentrations were also significantly lower ($p < 0.0001$) in men compared with the serum VEGF concentrations observed in women during the mid-follicular (3 ± 0.8 ng/ml) and in the late luteal phases (4.43 ± 0.9 ng/ml) of the menstrual cycle.

Table 12.1: Mean ± SD values of serum hormone measurements during the menstrual cycle.

Hormone value In serum	Early follicular phase	Preovulatory phase	Midluteal phase
FSH (iu/L)	4.57 ± 1	11.5 ± 5.2	NM
LH (iu/L)	4 ± 1.4	38.4 ± 9.8	NM
Oestradiol (pmol/l)	123.2 ± 44	779 ± 371	334 ± 173
Testosterone (nmol/l)	0.81 ± 0.5	NM	NM
Progesterone (nmol/l)	NM	NM	42.7 ± 18.3
VEGF (ng/ml)	2.44 ± 0.2	3 ± 0.8	4.43 ± 0.9

NM - Not measured

Discussion:

While changes in uterine and ovarian vascularity during the normal menstrual cycle, as demonstrated by pulsed and colour Doppler blood flow measurements, have been described previously (Scholtes et al., 1989, Steer et al., 1990, Sladkevicius et al., 1993), the factors which control them are not fully understood.

Following exposure of the dominant ovarian follicle to the mid-cycle surge of LH, blood vessels grow from the theca into the recently ruptured follicle to form a complex vascular network, a major feature of the development and differentiation of the corpus luteum (Pictures 12.8 a, b, c).

In this study it was observed that serum VEGF concentrations rose in the luteal phase of the menstrual cycle in parallel with changes in ovarian and uterine blood flow velocities. Although there was a close relationship between serum VEGF measurements and blood flow velocities (Fig. 12.5 - 12.7), the causal relationship between the factors is a matter for speculation. The fact that the rise in serum VEGF concentrations during the menstrual cycle remained significant after controlling statistically for changes in ovarian and uterine artery blood flow is consistent with change in VEGF, reflected in its serum concentrations, being one of the primary events that causes vascular growth.

VEGF is produced by the ovary immediately before (Ravindranath et al., 1992, Shweiki et al., 1993, Koos et al., 1995, Gordon et al., 1996) and after ovulation and is thought to mediate neovascularisation of the corpus luteum (Phillips et al., 1990, Yan et al., 1993, Koos et al., 1995, Kamat et al., 1995). These dynamic changes within the ovary were reflected in the peripheral venous concentrations of VEGF. Changes in VEGF during the normal menstrual cycle have also been demonstrated in histological sections of the human endometrium (Shifren et al., 1996). Further, positive correlations between serum VEGF and progesterone concentrations in the midluteal phase of the menstrual cycle were observed. This relationship could not be established in our larger series of patients undergoing IVF since serum progesterone concentrations after embryo transfer were not measured in patients undergoing IVF. Moreover, these patients received progesterone supplementation after embryo transfer, which means that in this situation serum progesterone concentrations do not reflect corpus luteum activity.

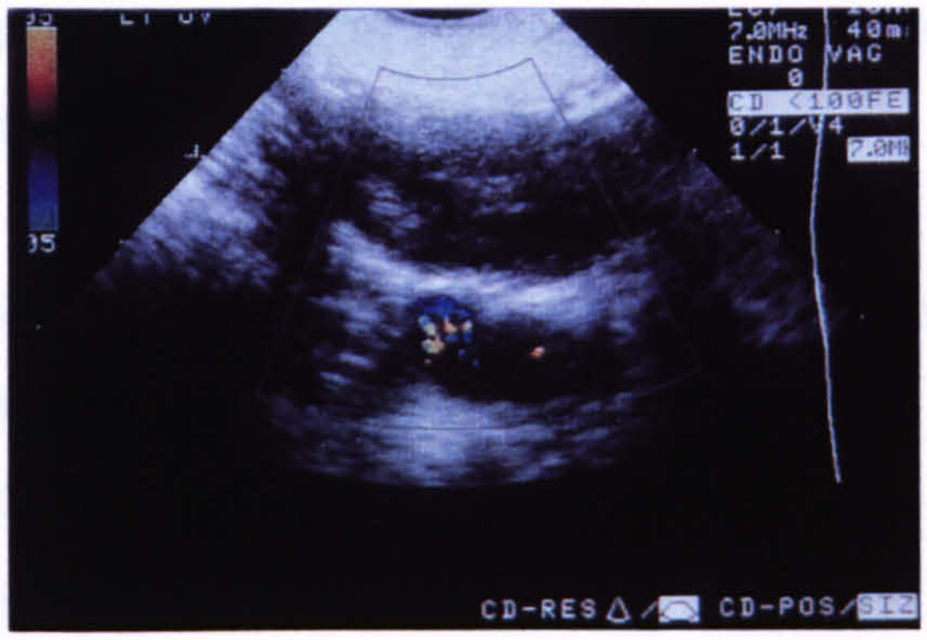


Figure 12.8 (a)

Colour Doppler blood flow velocity in the ovarian stroma of a normal ovary during the early follicular phase of the menstrual cycle.

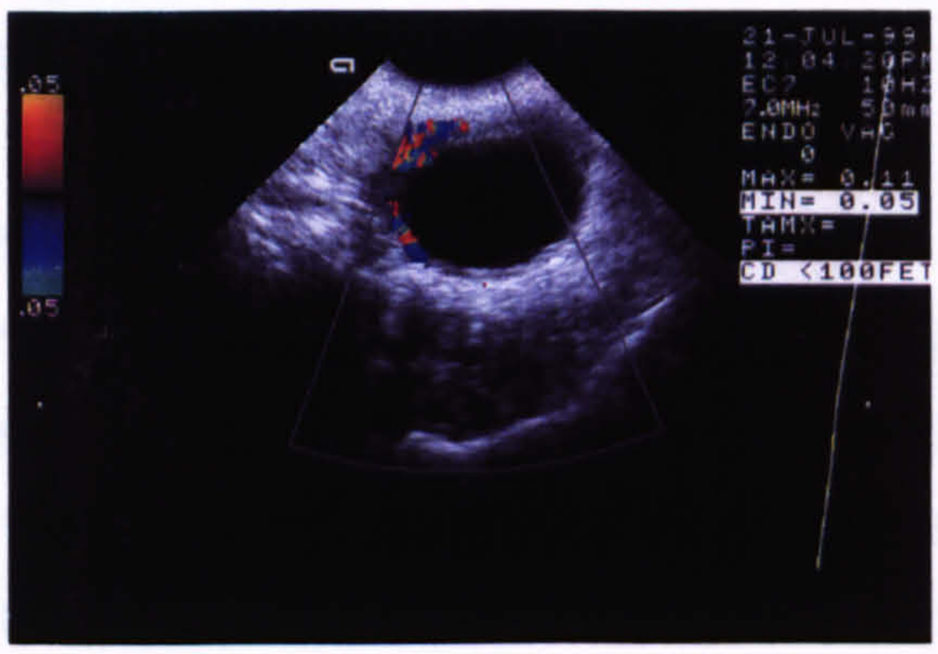


Figure 12.8 (b)

Colour Doppler blood flow in the ovarian stroma around the preovulatory follicle. Follicular vessels are seen developing as an intense ring of colour circumferentially around the dominant follicle during the preovulatory phase of the menstrual cycle.

Figure 12.8 (c)

Colour Doppler blood flow in the ovarian stroma during the midluteal phase of the menstrual cycle. Intense localisation of colour in the corpus luteum is seen in the mid-luteal phase of the menstrual cycle.

The rise in serum VEGF concentrations in the luteal phase of the menstrual cycle would be consistent with the reproductive tract making a major contribution to circulating VEGF concentrations.

Although adult organs (e.g., liver, lung, breast and carcinomas) do express VEGF mRNA (Ferrara and Davis-Smith, 1997), there is no evidence in the literature to suggest that adult organs contribute to serum VEGF concentrations, cyclically or acyclically.

The Doppler blood flow findings reported here are consistent with those published previously (Goswamy et al., 1988, Steer et al., 1990, Battaglia et al., 1990, Santolaya Forgas, 1992, Sladkevicius et al., 1993, Campbell et al., 1993, Bourne et al., 1996, Lunenfeld et al., 1996).

VEGF is expressed throughout the smooth muscle of the uterus. VEGF gene expression within the uterus is rapidly stimulated by oestradiol (Charnock - Jones et al., 1993, Cullinan - Bove and Koos, 1993, Hyder et al., 1996). It is possible, therefore that VEGF mediates the oestrogen induced increase in uterine vascular permeability and growth (Charnock - Jones et al., 1993, Cullinan - Bove and Koos, 1993, Hyder et al., 1996). The positive correlation of serum VEGF and oestradiol reported here is consistent with this finding. VEGF mRNA in normal myometrium and endometrium are significantly higher in the secretory phase than in the proliferative phase of the menstrual cycle (Li et al., 1994, Harrison -Woolrych et al., 1995, Torry et al., 1996). VEGF may be the principal factor that promotes the vascular growth, maintenance and hyperpermeability required for adequate receptivity of the cycling human endometrium.

A positive correlation was observed between serum VEGF and testosterone concentrations but not with LH concentrations. These data are consistent with the hypothesis that testosterone and not LH (prior to ovulation), may up-regulate VEGF gene expression. LH does however, up-regulate VEGF gene expression at/after ovulation. However this relation was not observed in patients who underwent IVF treatment. There is also evidence to support

the suggestion that testosterone up-regulates VEGF expression within the prostate gland after castration (Franck-Lissbrant et al 1998) but whether it up-regulates ovarian VEGF expression is not known.

No differences were observed in VEGF concentrations between healthy men and women who were in the early follicular phase of the menstrual cycle. However serum VEGF concentrations were significantly higher in women during the mid-follicular and the luteal phases of the menstrual cycle compared with men. This can be explained by the absence of cyclical changes within the reproductive tract of men. There is evidence to suggest that testosterone induces VEGF production within the male reproductive tract (Frank-Lissbrant et al., 1998). Further studies are needed however, performed on hypogonadal men before and during testosterone treatment, to investigate the hypothesis that testosterone stimulates production of VEGF in men.

Study 2: Serum vascular endothelial growth factor (VEGF) concentrations and ovarian blood flow in women with normal and polycystic ovaries.

Patients in this study comprised the first 60 of the 107 patients recruited for the IVF study. 36 women had normal ovaries, 14 had PCO and 10 had PCOS. Demographic data from the 3 study groups were similar although women with PCOS had a significantly higher mean BMI ($p < 0.001$) than women with normal ovaries and those with PCO (Table 12.2).

Table 12.2: Demographic data of the three groups of women in the PCO study.

Variables	Normal ovaries (n = 36)	Polycystic ovaries (n = 14)	Polycystic ovary syndrome (n = 10)
Mean age (range)	36.2 (28 - 43)	35.2 (25 - 40)	33.1 (28 - 41)
BMI (mean ± SD)	22.9 ± 2.8	24 ± 4.4	**26.9 ± 2.2
Parous women n(%)	5 (8.3%)	5 (8%)	4 (6%)
Duration of infertility	6.8 ± 3.1	7 ± 3.8	6.7 ± 2.7
Causes of infertility Male factor	10 (27.7%)	5 (35.7%)	3 (30%)
Tubal factor	6 (16.6%)	3 (21.4%)	2 (20%)
Endometriosis	7 (19.4%)	3 (21.4%)	2 (20%)
Unexplained	8 (22.2%)	3 (21.4%)	3 (30%)

** Statistically significant difference ($p = 0.001$) between women with normal ovaries and PCO compared with women with PCOS.

Serum VEGF and hormone concentrations obtained from the three groups of women is shown in Table 12.3. Mean serum VEGF concentrations in women with PCO and PCOS were significantly higher ($p < 0.0001$) than in women with normal ovaries (Fig 12.9). There were no statistically significant differences in serum VEGF concentrations between women with PCO and PCOS. Significantly higher LH and testosterone concentrations were found in women with PCOS than in the other two groups (Table 12.3).

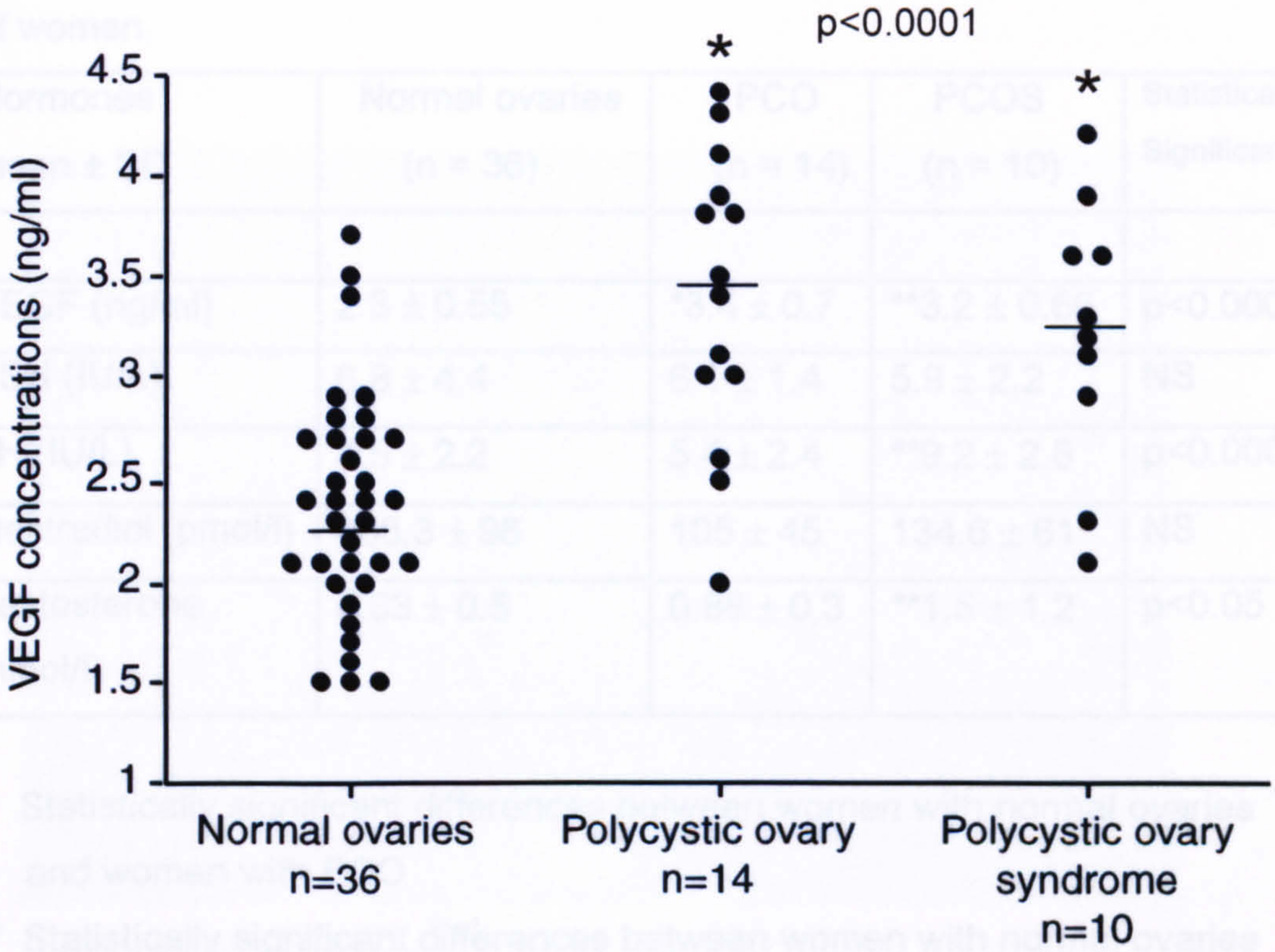


Figure 12.9

Serum VEGF concentrations in 60 women with normal ovaries, PCO and PCOS. *Significantly higher in PCO and PCOS (*p<0.0001) compared with normal ovaries.

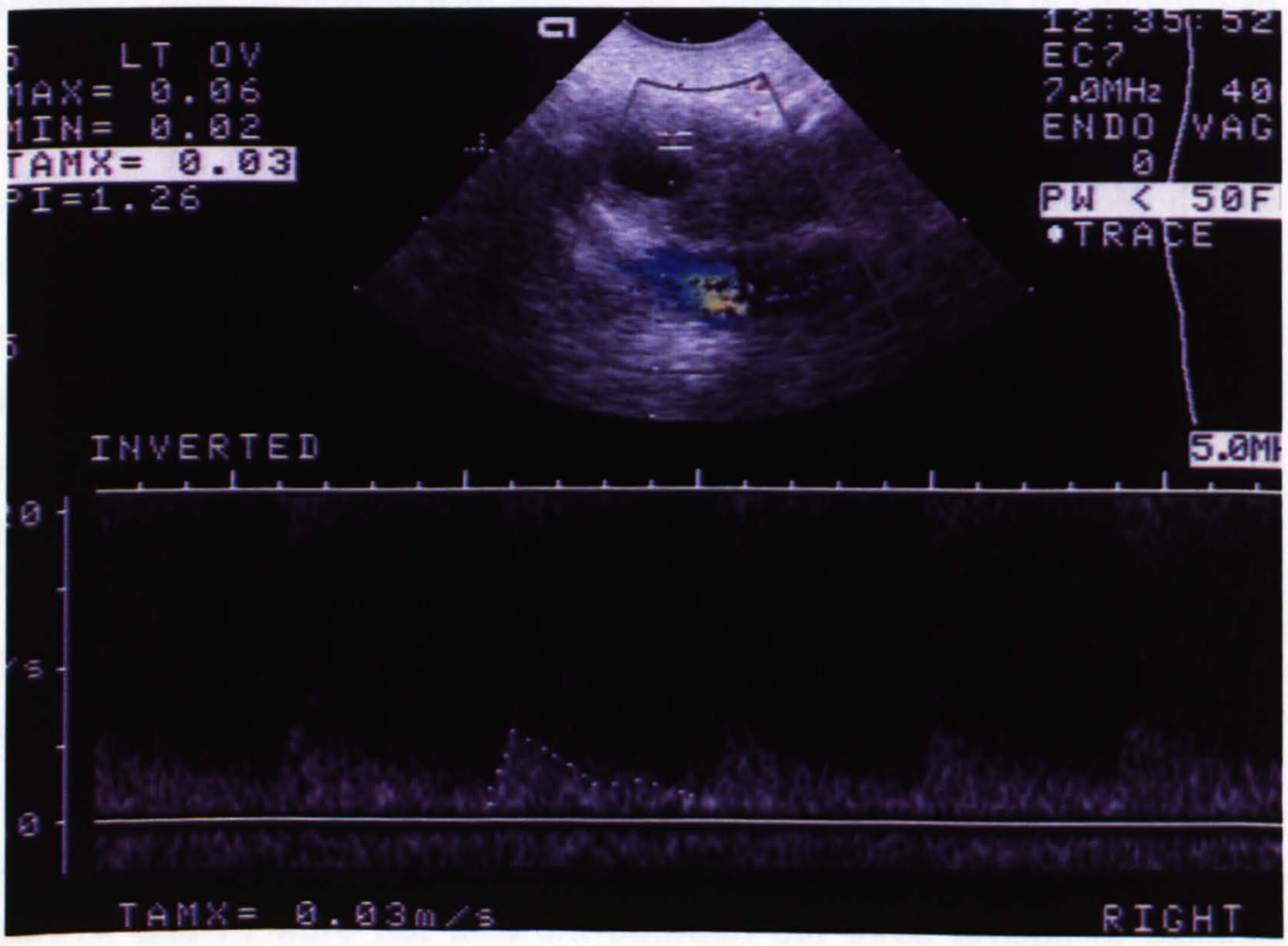


Figure 12.10(a)

Doppler blood flow velocities in the ovarian stroma of women with normal ovaries.The peak systolic velocity, TAMXV and the pulsatility index of the blood flow are calculated.

Table 12.3: Serum VEGF and hormone concentrations in the three groups of women.

Hormones mean ± SD	Normal ovaries (n = 36)	PCO (n = 14)	PCOS (n = 10)	Statistical Significance
VEGF (ng/ml)	2.3 ± 0.55	*3.4 ± 0.7	**3.2 ± 0.66	p<0.0001
FSH (IU/L)	6.8 ± 4.4	6.1 ± 1.4	5.9 ± 2.2	NS
LH (IU/L)	4.5 ± 2.2	5.4 ± 2.4	**9.2 ± 2.8	p<0.0001
Oestradiol (pmol/l)	158.3 ± 96	105 ± 45	134.6 ± 61	NS
Testosterone (nmol/l)	0.83 ± 0.5	0.88 ± 0.3	**1.5 ± 1.2	p<0.05

* Statistically significant differences between women with normal ovaries and women with PCO

** Statistically significant differences between women with normal ovaries and women with PCOS

Recordings of blood flow velocity waveforms from the ovarian stroma were possible in 98% of women with polycystic ovaries and in 88% in women with normal ovaries. Analyses of Doppler blood flow velocities of ovarian stromal blood vessels showed no significant differences in the PSV, TAMXV, PI or RI between the right and left ovaries. Mean values of the two blood flow velocity measurements were therefore calculated and used for subsequent analyses. Subjective assessment of the ovaries demonstrated a larger number of blood vessels and a greater intensity of colour blood flow within the ovarian stroma of women with PCO and PCOS compared with those in women with normal ovaries (12.10 a, b1, b2).

PSV and TAMXV were significantly higher in women with PCO and PCOS compared with those in women with normal ovaries (Table 12.4) (Fig 12.11, 12.12). There were no significant differences in the blood flow velocities (PSV and TAMXV) of women with PCO compared with women with PCOS. Mean RI and PI were not different between the three groups of patients (Table 12.4).

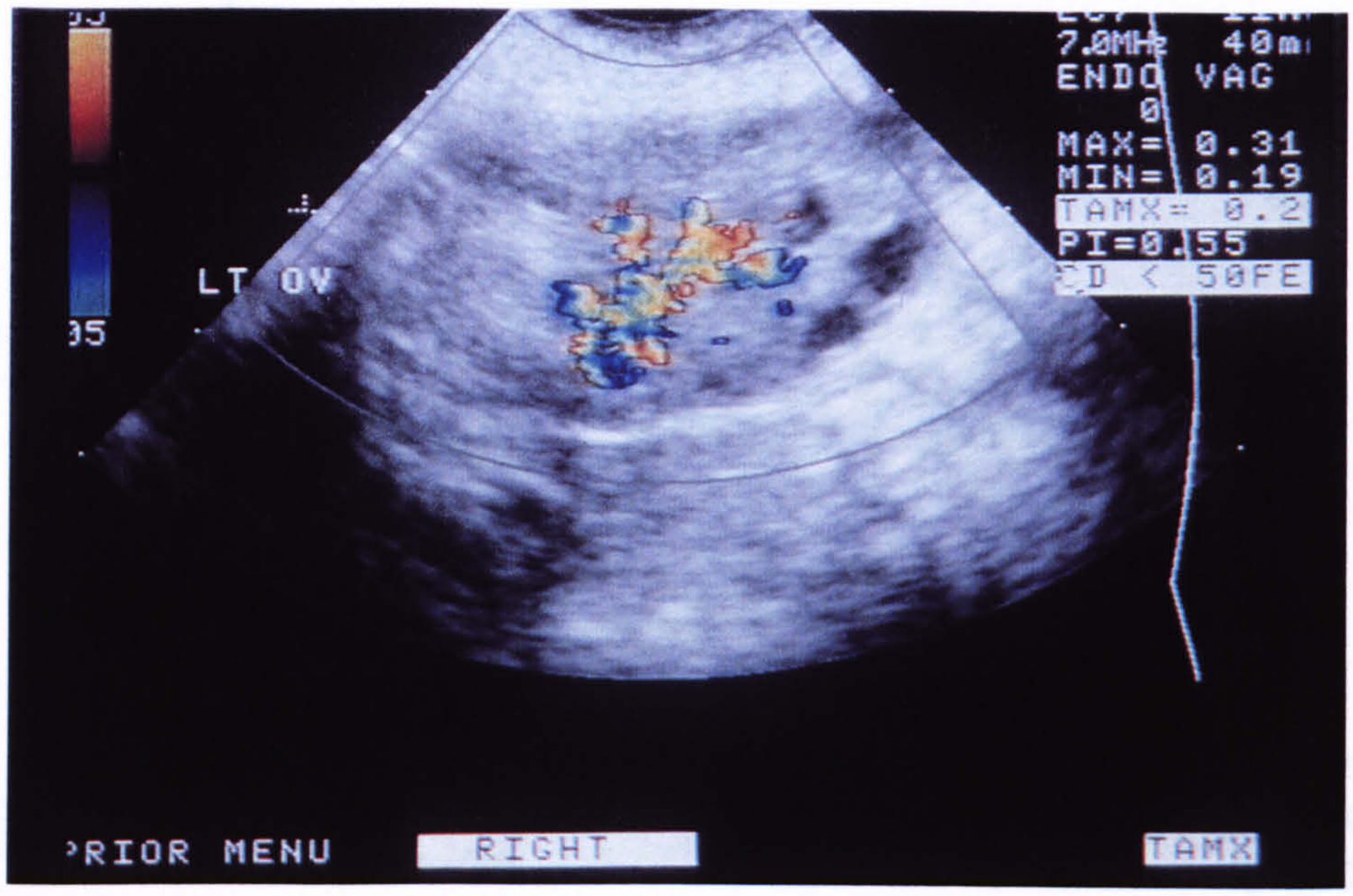


Figure 12.10 (b1)

Colour Doppler blood flow observed within the ovarian stroma of a polycystic ovary during the early follicular phase of the menstrual cycle.

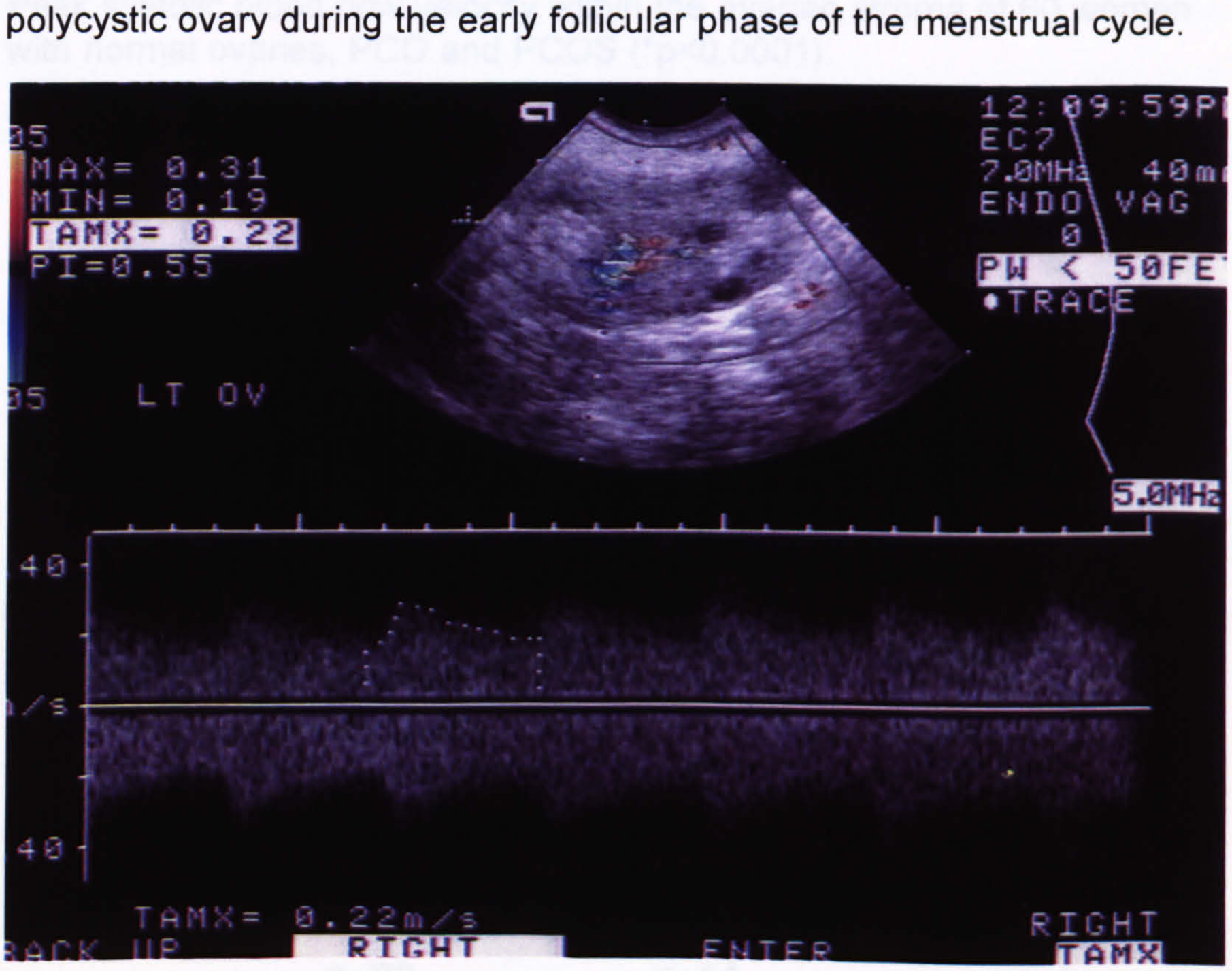


Figure 12.10.(b2)

Colour doppler blood flow velocities within the ovarian stroma of women with PCO during the early follicular phase of the menstrual cycle.
The PSV, TAMXV and the pulsatility index of the blood flow are calculated.

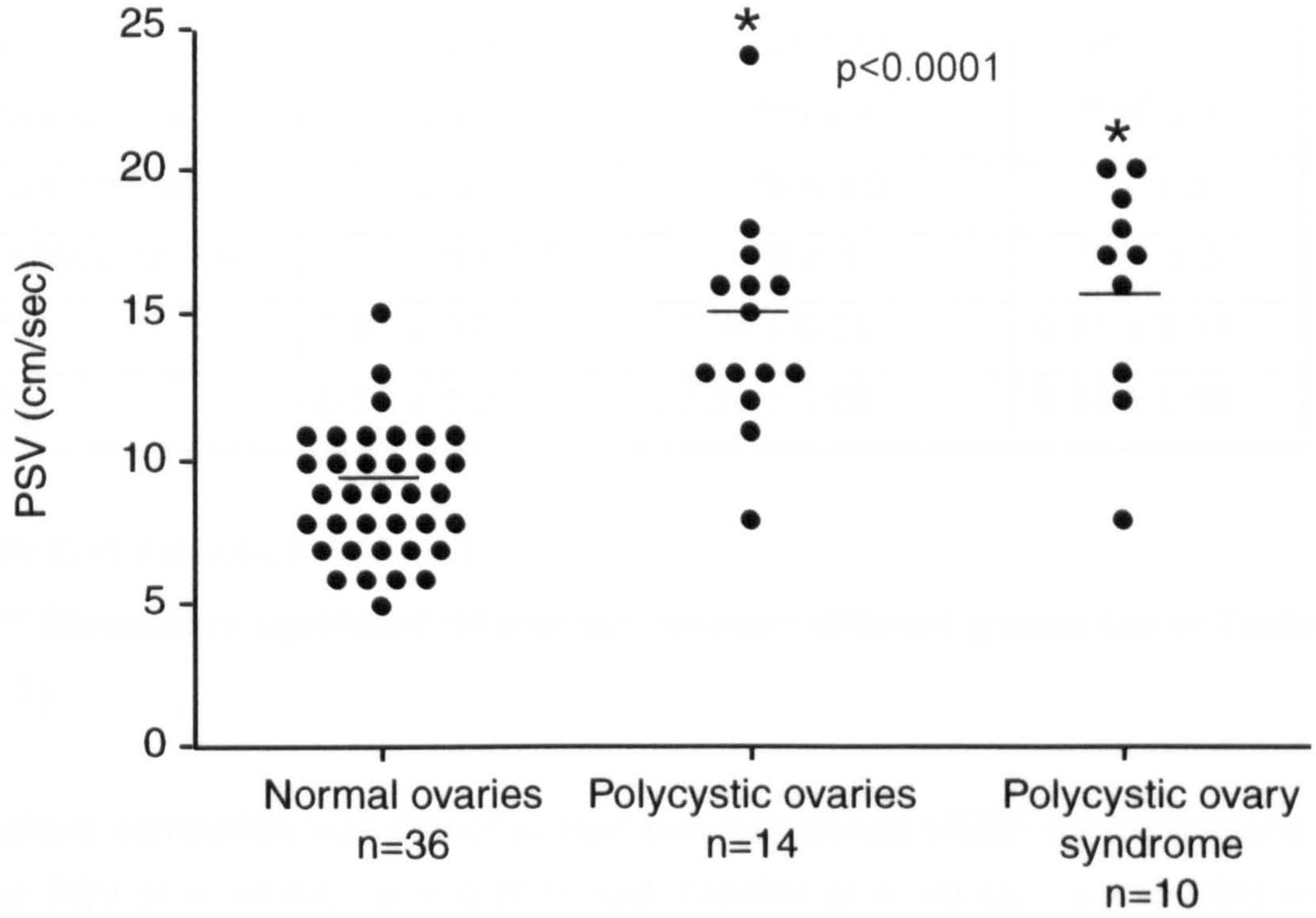


Figure 12.11
Peak systolic blood flow velocity within the ovarian stroma of 60 women with normal ovaries, PCO and PCOS (*p<0.0001).

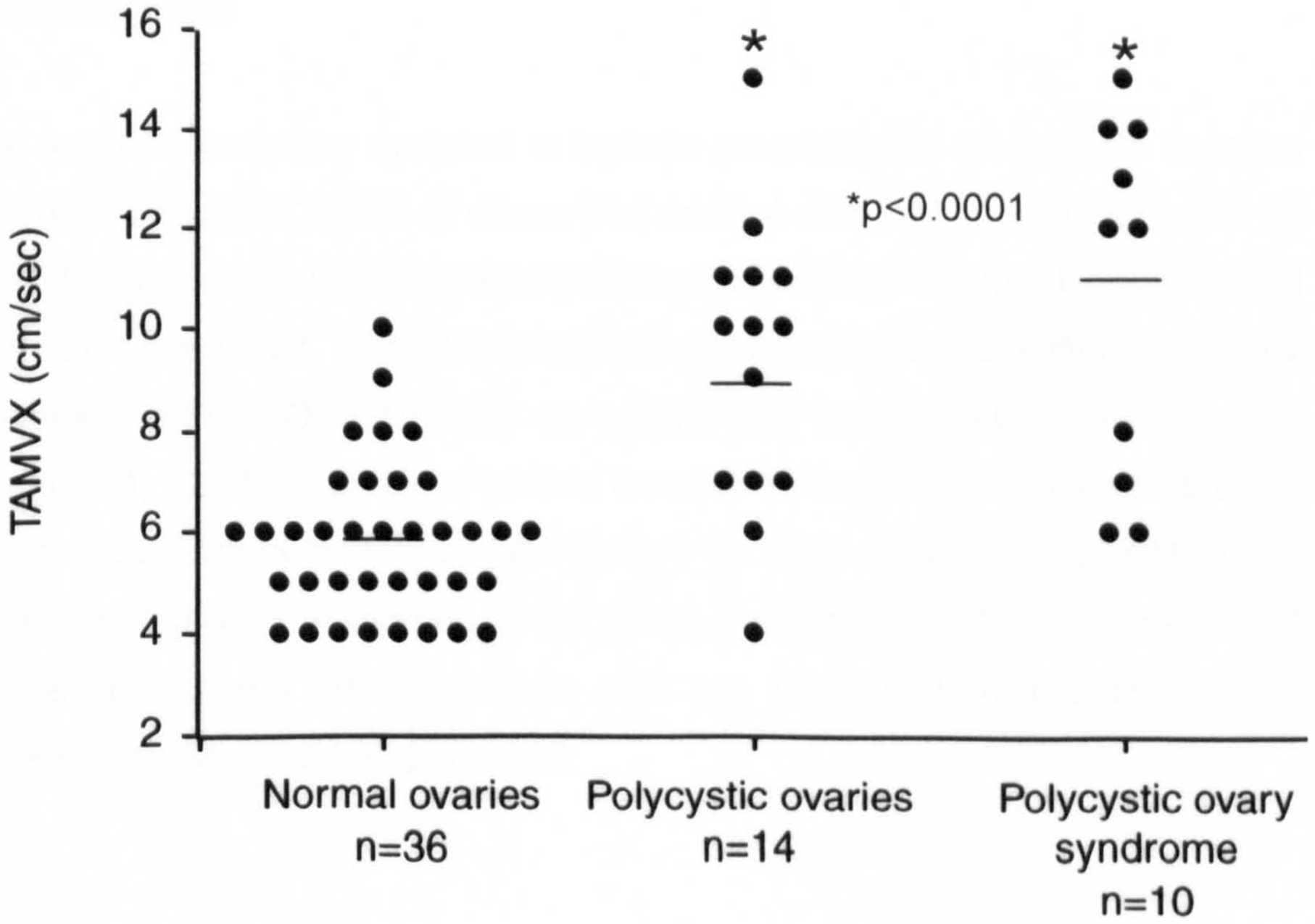


Figure 12.12

Time averaged maximum blood flow velocity within the ovarian stroma of 60 women with normal ovaries, PCO and PCOS (*p<0.0001).

Table 12.4: Doppler blood flow indices in three groups of women.

Blood flow indices mean ± SD	Normal ovaries (n = 36)	PCO (n = 14)	PCOS (n = 10)
PSV cm/sec	9 ± 2	*15 ± 4	**16 ± 4
EDV cm/sec	3 ± 1	*6.4 ± 3	**7 ± 3
TAMXV cm/sec	5.8 ± 1.5	*9 ± 3	**11 ± 3
PI	0.95 ± 0.021	0.94 ± 0.25	0.81 ± 0.19
RI	0.57 ± 0.07	0.57 ± 0.08	0.54 ± 0.09

EDV End diastolic flow.(V_{min})

*, ** Statistically significant differences between different groups (as in Table 12.3).

Positive correlation was demonstrated between serum VEGF concentrations and PSV ($r = +0.44$, $p < 0.001$) and TAMXV ($r = +0.45$, $p < 0.001$) in women in all three groups (Fig 12.13). No correlations of VEGF concentrations with serum FSH, LH, oestradiol or testosterone concentrations were observed. There were no correlation of serum hormone concentrations and peak blood flow velocities measured on day 2 or 3 of the menstrual cycle.

The analysis was later updated to include an additional 47 patients to make a total of 107 patients, 65 of whom had normal ovaries, 29 had PCO and 15 had PCOS. Serum VEGF concentrations and ovarian stromal Doppler blood flow velocities (PSV, TAMXV) remained consistently and significantly higher in women with PCO and PCOS compared with women with normal ovaries (Fig 12.14, 12. 15, 12.16). Positive correlation was demonstrated between serum VEGF concentrations and PSV ($r = +0.36$, $p < 0.001$) and TAMXV ($r = +0.41$, $p < 0.001$) in women in all three groups (Fig 12.17). These 107 women underwent IVF treatment and are described in the section on Material and Methods on page 105.

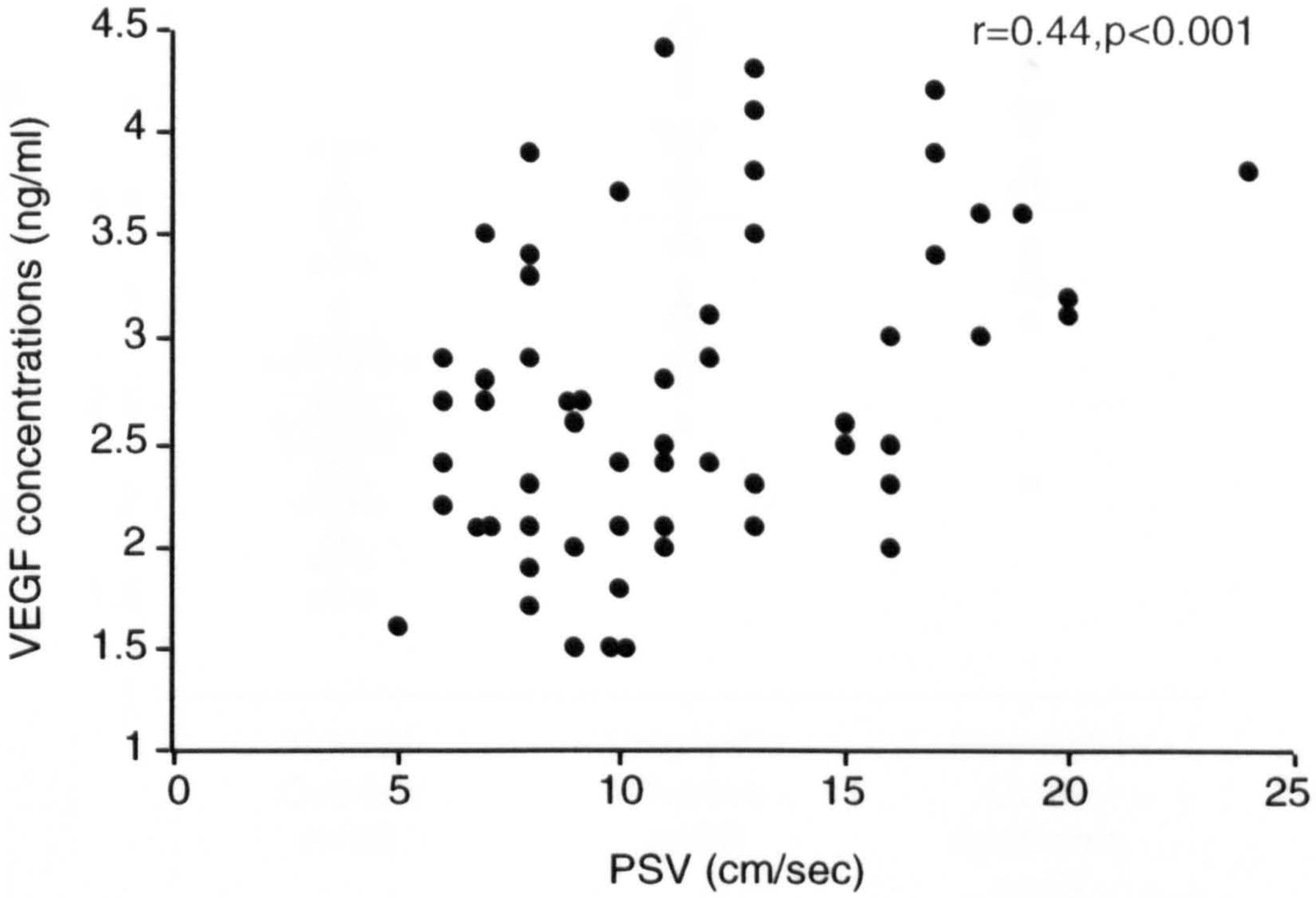


Figure 12.13

Pearson's correlation coefficient between serum VEGF concentrations and Doppler blood flow velocity within the ovarian stroma of 60 women with normal ovaries, PCO and PCOS ($r=0.44$, $p<0.001$).

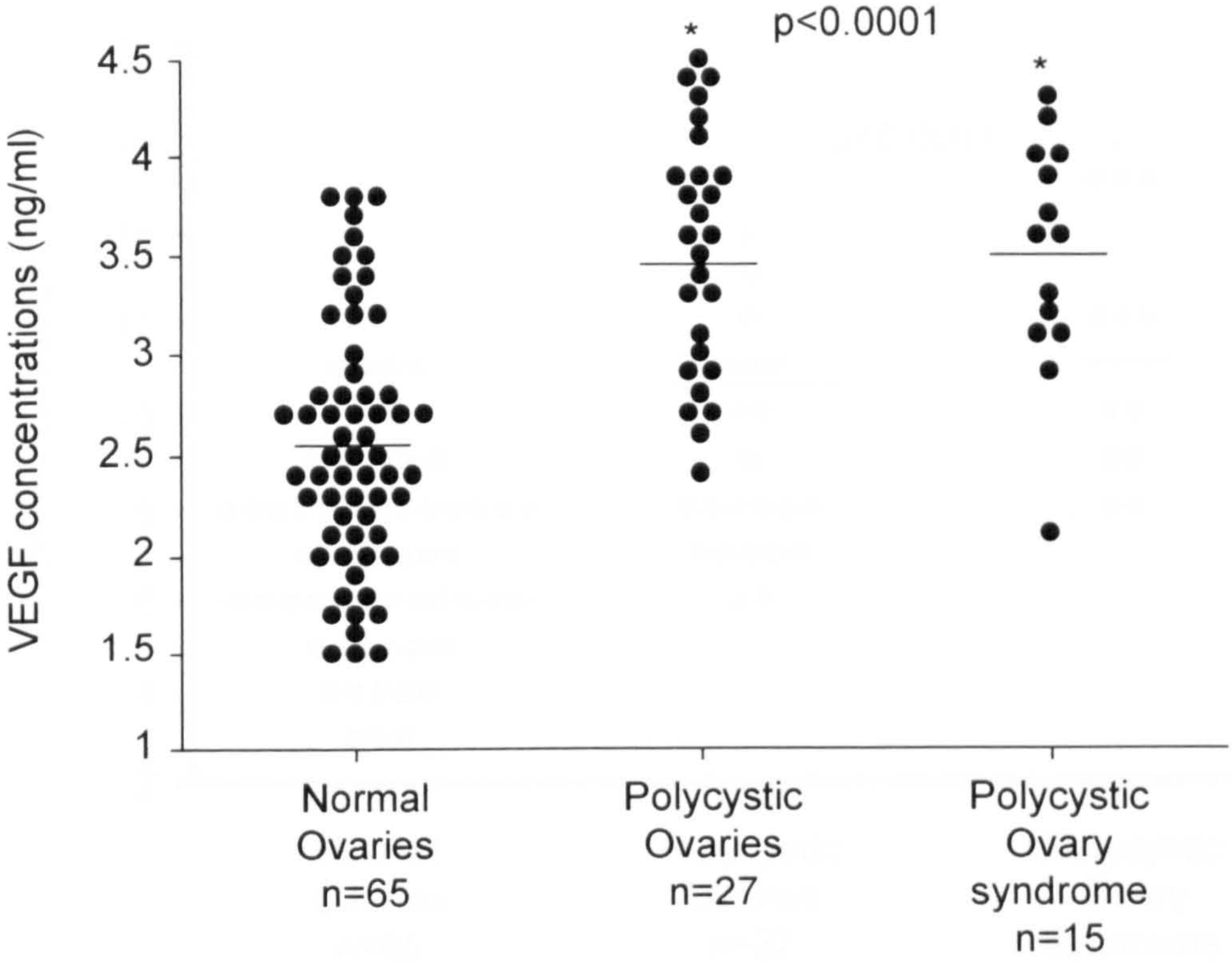


Figure 12.14

Serum VEGF concentrations in 107 women with normal ovaries, PCO and PCOS. *Significantly higher in PCO and PCOS (*p<0.0001) compared with normal ovaries.

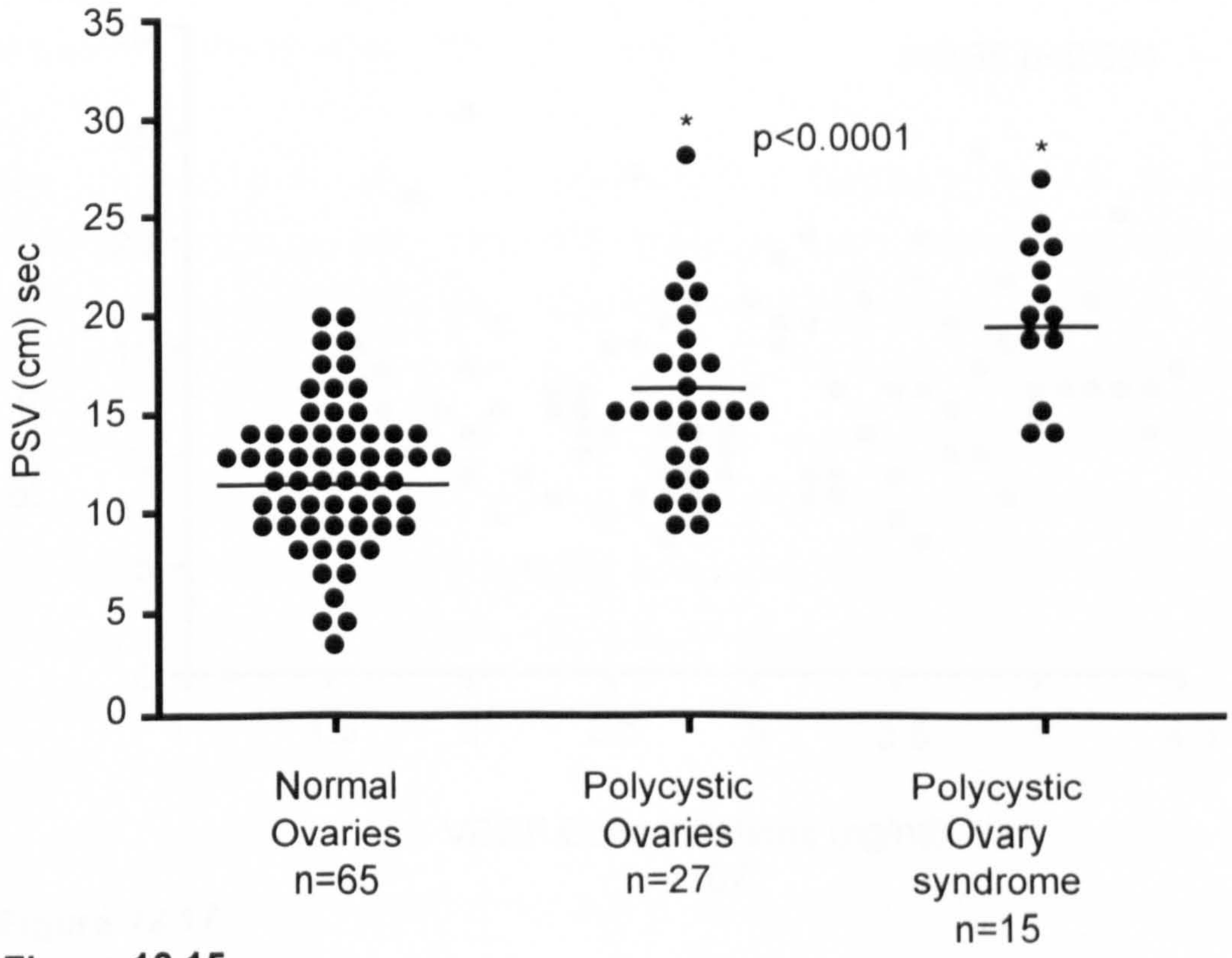


Figure 12.15

Peak systolic blood flow velocity within the ovarian stroma of 107 women with normal ovaries, PCO and PCOS (*p<0.001).

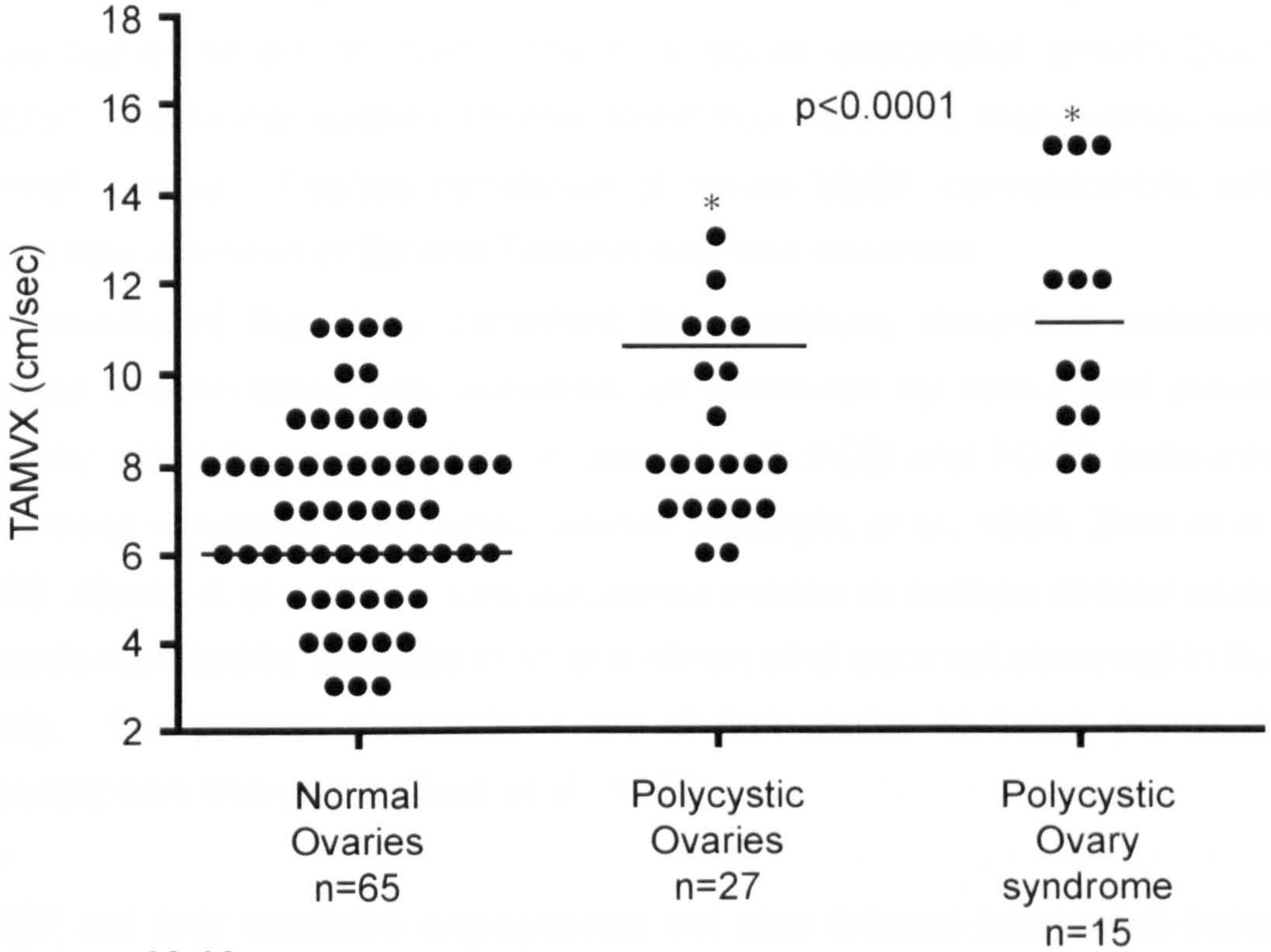


Figure 12.16

Time averaged maximum blood flow velocity within the ovarian stroma of 107 women with normal ovaries, PCO and PCOS (*p<0.0001).

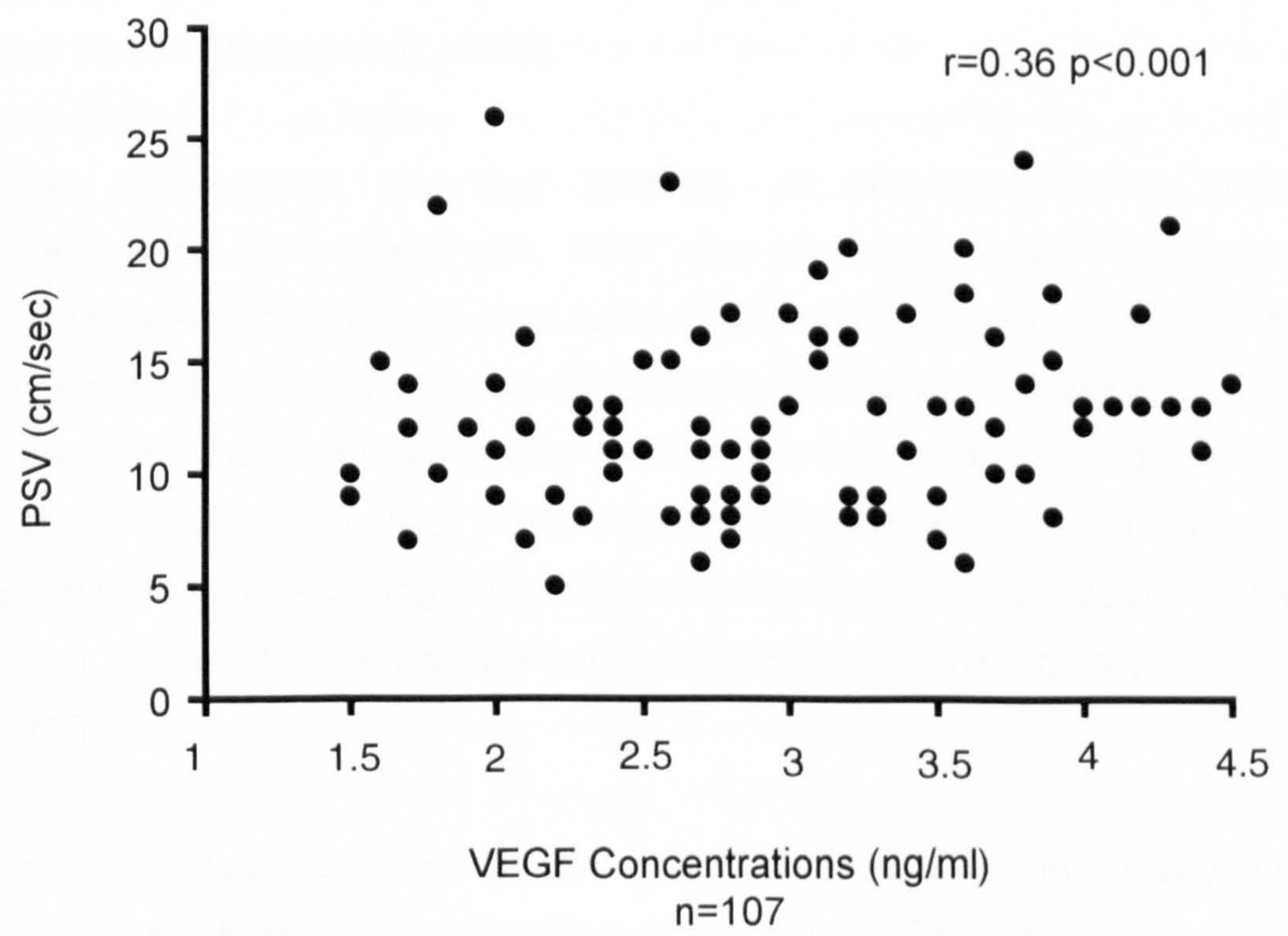


Figure 12.17

Pearson's correlation coefficient between serum VEGF concentrations and Doppler blood flow velocity within the ovarian stroma of 107 women with normal ovaries, PCO and PCOS (r=0.36, p<0.001).

Discussion:

The important findings of this study are that women with polycystic ovaries have higher serum concentrations of vascular endothelial growth factor (VEGF) and higher ovarian stromal blood flow velocities than women with normal ovaries. Positive correlation of serum VEGF concentrations with blood flow velocities (PSV and TAMXV) was also observed.

The results of this study confirmed the previously described increased ovarian stromal blood flow velocities, as measured by colour and pulsed Doppler blood flow examination, in women with PCO and PCOS compared with those in women with normal ovaries (Battaglia et al., 1995; Zaidi et al., 1995; Aleem et al., 1996). Low resistance indices in ovarian stromal blood vessels reported by Battaglia et al. and Aleem et al were not observed in this study. Our present observations are in fact similar to those previously reported from this centre (Zaidi et al., 1995).

VEGF not only mediates angiogenesis but also induces connective tissue stromal growth by increasing microvascular permeability, which leads to extravasation of plasma proteins. The extravascular matrix thus formed favours ingrowth of new blood vessels and fibroblasts, which in turn organise the avascular provisional fibrin matrix into a mature, vascularised connective tissue stroma (Kamat et al., 1995).

These observations and our findings of elevated serum VEGF concentrations in women with PCO may in part explain the dense hyperechogenic and highly vascularised stroma of polycystic ovaries, as demonstrated by Doppler blood flow studies in this and previous studies. Perhaps the increased vascularity results from over-expression of ovarian VEGF in women with PCO. This hypothesis would be supported by the demonstration of a strong immunohistochemical staining of VEGF in the ovarian stroma of three patients with polycystic ovary syndrome (Kamat et al., 1995).

It is well established that growth factors are involved in intraovarian regulatory mechanisms. Insulin like growth factor-1 (IGF-1) has been shown

to induce LH receptors and LH mediated angiogenesis has been previously described (Findlay et al., 1986). Perhaps increased intraovarian concentrations of VEGF are caused by increased secretion and pulsatility of LH, an important pathophysiological feature of PCOS. There are however large fluctuations of LH concentrations in women with PCOS over time (Adashi et al., 1995). Moreover raised LH concentrations are seen in only 40% of women with PCOS (Conway et al., 1989; Balen et al., 1995) and by definition are not present in women with PCO but not PCOS.

In addition in the present study, elevated concentrations of VEGF and increased stromal blood flow were found in women with PCO as well as those with PCOS, consistent with increased VEGF production being a constitutive feature of the PCO. Further, no fall in serum VEGF concentrations after pituitary desensitisation with GnRH analogues was observed, (Fig 12.18). This reduces serum LH concentrations. Moreover there was no correlation of serum VEGF with LH during the early follicular phase of the menstrual cycle. It therefore seems unlikely that LH mediates the increased vascularity and increased VEGF expression in the stroma of polycystic ovaries.

It was next considered whether increased ovarian circulating androgens in women with PCO could be a cause of the increased vascularity. In support, there are recent data indicating exposure to testosterone increased VEGF expression within the prostate gland and castration decreased testicular and prostatic VEGF (Frank-Lissbrant et al., 1998). Perhaps ovarian hyperandogenism in women with PCO increases VEGF expression. This over-expression may however, be a constitutive feature of PCO. For example, women with hypogonadotrophic hypogonadism with PCO are known to overrespond to treatment (Shoham et al., 1992).

Unlike other growth factors responsible for angiogenesis, e.g. bFGF, which is largely intracellular and nondiffusible, VEGF, is a soluble diffusible growth factor. There is also evidence that angiogenic factors like bFGF, TGF beta, PDGF and nitric oxide act as agonists to the action of VEGF within the ovary

(Connolly et al., 1991).

Oestradiol (E_2) is a moderator of uterine and ovarian vascularity (de Ziegler et al., 1991; Steer et al., 1990). However visualisation of distinct ovarian stromal blood vessels in PCO in the early follicular phase of the menstrual cycle shows a striking difference in the vascular pattern from normal ovaries. It is unlikely that E_2 causes this difference, particularly since its concentrations are not raised in women with PCO.

It is well known that the risk of OHSS in programmes of ovulation induction and IVF is higher in women with PCO compared with women with normal ovaries (Rizk et al., 1991, MacDougall et al., 1993). This finding again might be the result of overexpression of VEGF in women with PCO, who characteristically recruit excess number of follicles with even small doses of gonadotrophin stimulation. Increased vascularity caused by the increased amount of VEGF within the stroma may result in a failure of diversion of blood flow away from cohort follicles to the leading follicle, thereby permitting uninhibited growth of other follicles and a multifollicular response.

Elevated concentrations of VEGF in various body fluids e.g.; ascitic fluid, follicular fluid, serum and urine have recently been established in women undergoing ovarian stimulation who develop OHSS (McClure et al., 1994; Robertson et al., 1995; Krasnow et al., 1996; Abramov et al., 1997). Our present findings provide a mechanism that helps to explain the link between VEGF, OHSS and PCO.

Study 3: Changes in serum vascular endothelial growth factor (VEGF) and Doppler blood flow velocities in ovarian and uterine blood vessels during IVF treatment cycles and its relevance to ovarian hyperstimulation syndrome and polycystic ovaries.

During ovarian stimulation prior to IVF treatment, serum VEGF concentrations rose from 2.93 ± 0.77 ng/ml in the early follicular phase of the menstrual cycle to 3.6 ± 0.91 ng/ml on the day of hCG administration (p < 0.0001) (Fig 12.18). No change was detected between the early follicular phase and after 14 days of pituitary desensitisation with GnRH analogue, Buserelin (3.0 ± 0.7 ng/ml). Thereafter serum VEGF rose to 4.6 ± 0.93 ng/ml on the day of egg collection (p < 0.0001) and to 4.88 ± 1.02 ng/ml on the day of embryo transfer (p < 0.0001).

Doppler blood flow velocities (PSV and TAMXV) within the ovarian stromal and uterine blood vessels, rose in parallel with the rising serum VEGF concentrations (Fig 12.18). Within ovarian stromal blood vessels, no changes were observed in PI and RI but within uterine blood vessels, PI and RI decreased gradually until the day of egg collection and further decreased after egg collection.

Women who developed OHSS (n = 20) had consistently higher serum VEGF concentrations before and throughout their IVF cycle, except in the phase after pituitary desensitisation but before gonadotrophin stimulation was commenced (Fig 12.19). The rise of serum VEGF from the day of hCG administration to the day of egg collection was greater in women who developed OHSS compared with that in women who did not develop OHSS (n = 87) (Fig 12.19).

Within the ovarian stromal blood vessels, Doppler blood flow velocities (PSV and TAMXV) were higher in women who developed OHSS after gonadotrophin stimulation was commenced compared with women who did not (Fig 12.19). There were no differences detected however, between the PI and RI within the ovarian stromal blood vessels and no differences

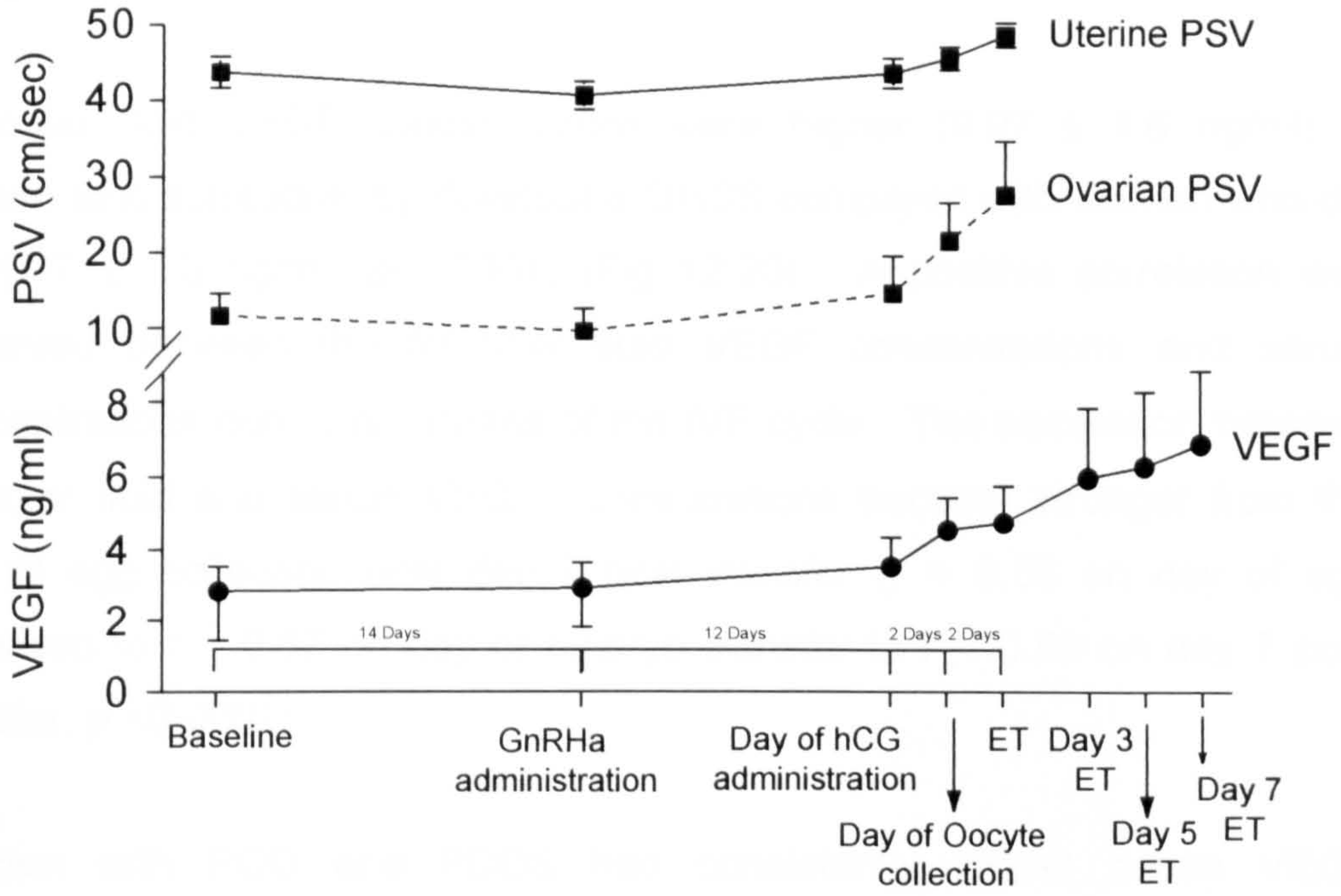


Figure 12.18

Changes in serum VEGF concentrations and Doppler blood flow velocities (PSV) within the ovarian stromal and uterine blood vessels during 107 IVF cycles.The X axes in figures 12.16 and 12.19 have variable time scales in order to depict the interval between various points, eg between baseline to GnRHa administration is equal to14 days and between day of hCG administration and oocyte collection is equal to 2 days.

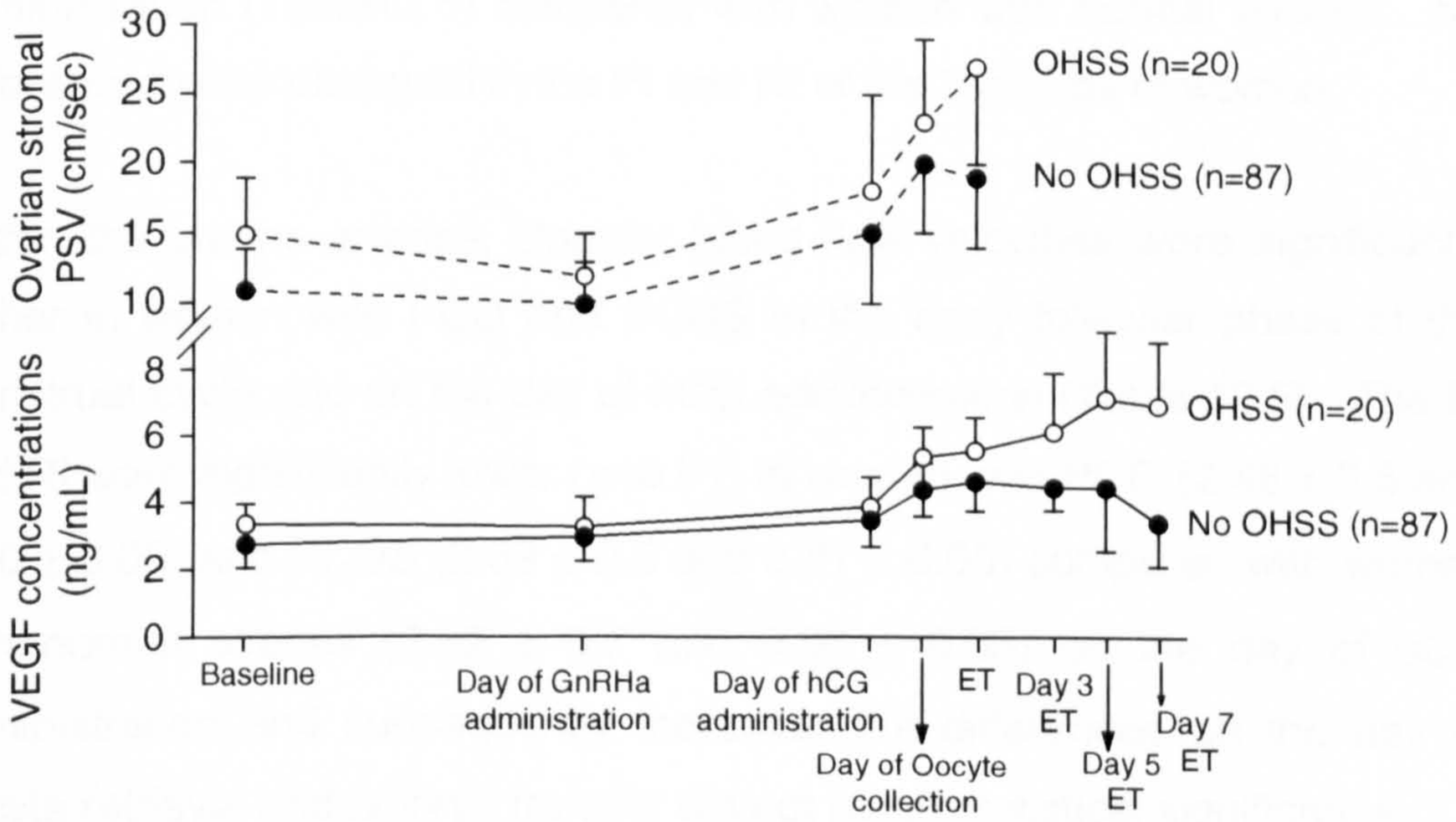


Figure 12.19

Changes in serum VEGF concentrations and Doppler blood flow velocities (PSV) in ovarian stroma during IVF cycles in women who developed OHSS (n=20) and in those who did not develop OHSS (n=87). Serum VEGF concentrations rose after hCG administration (*p<0.0001).

detected in blood flow velocities within the uterine arteries between the two groups of women.

Follicular fluid VEGF concentrations were higher (9.97 ± 1.6 ng/ml) in women who subsequently developed OHSS compared with women who did not (7.7 ± 0.6 ng/ml, $p<0.0001$) (Fig 12.20). A positive correlation was observed between the follicular fluid VEGF concentrations and serum concentrations during all phases of the IVF cycle. The correlation between follicular fluid and serum VEGF concentrations became stronger from the day of egg collection until day 7 post transfer (r = 0.58 on day of egg collection to r = 0.67 on day of embryo transfer to r = 0.89 on day 7 post transfer, p <0.0001)

Women with PCO and PCOS had consistently higher serum VEGF concentrations before and throughout their IVF cycle compared with women with normal ovaries (Fig 12.21). The differences remained significant even after excluding patients who developed OHSS (Fig 12.22).

Within the ovarian stromal blood vessels, Doppler blood flow velocities (PSV and TAMXV) were significantly higher in women with PCO and PCOS during the early follicular phase of the menstrual cycle and on the day of hCG administration (Table12.5) compared with women with normal ovaries. No differences were observed in the PI and RI of the 3 groups of women.

Within the uterine arteries, Doppler blood flow velocities were significantly higher in women with PCO and PCOS in the early follicular phase of the menstrual cycle and on the day of hCG administration (Table 12.5). The PI and RI were significantly lower ($p<0.01$) in women with PCO (2.48 ± 0.5 and 0.80 ± 0.05) and PCOS (2.59 ± 0.5 and 0.81 ± 0.05) compared with women with normal ovaries (2.82 ± 0.7 and 0.86 ± 0.05) on the day of hCG administration and subsequently thereafter but differences on the day of oocyte retrieval and embryo transfer did not reach statistical significance.

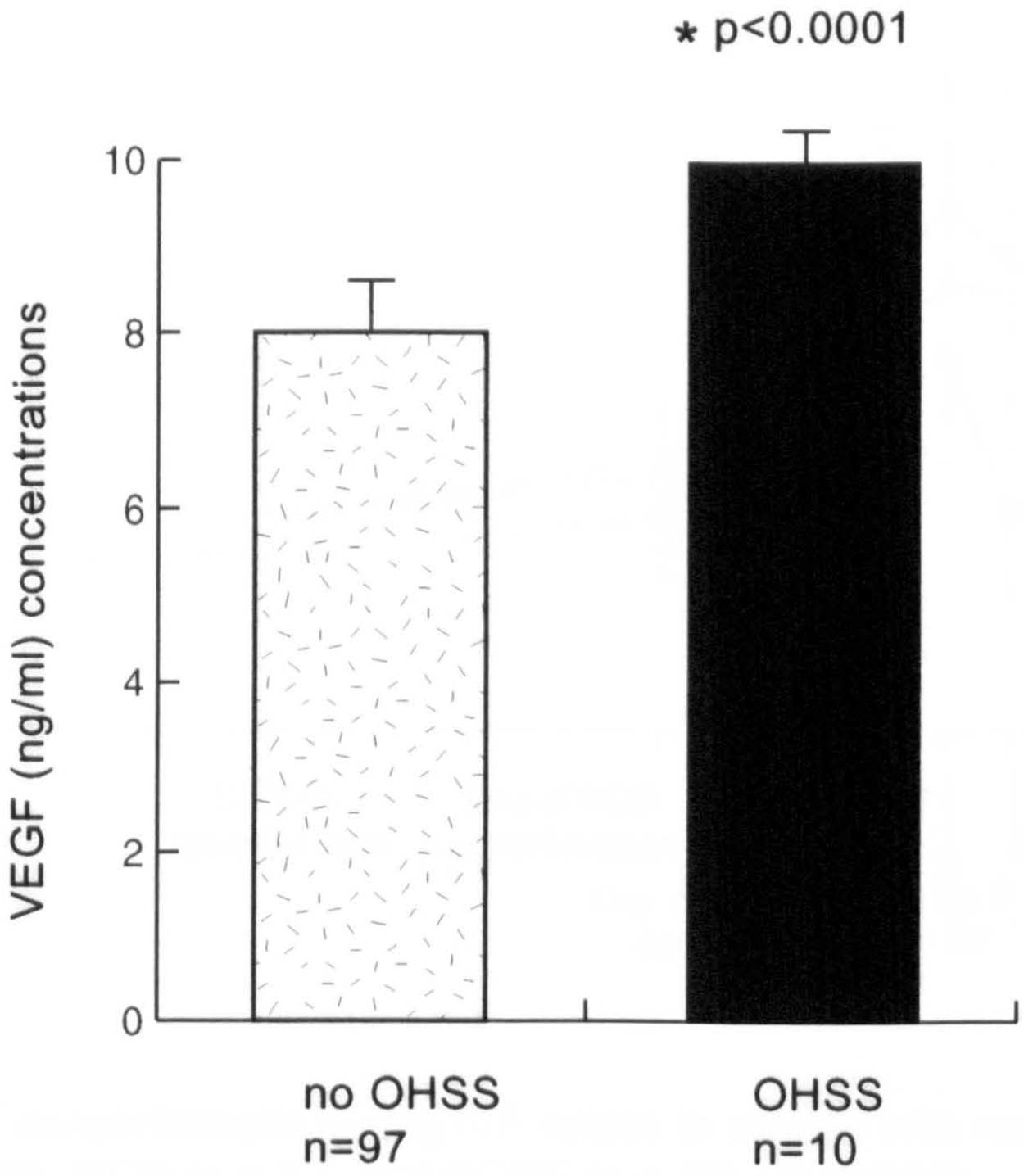


Figure 12.20

Follicular fluid VEGF concentrations in women who developed severe OHSS (n=10) and in those who did not develop OHSS (n=97), after controlling for PCO (*p<0.0001).

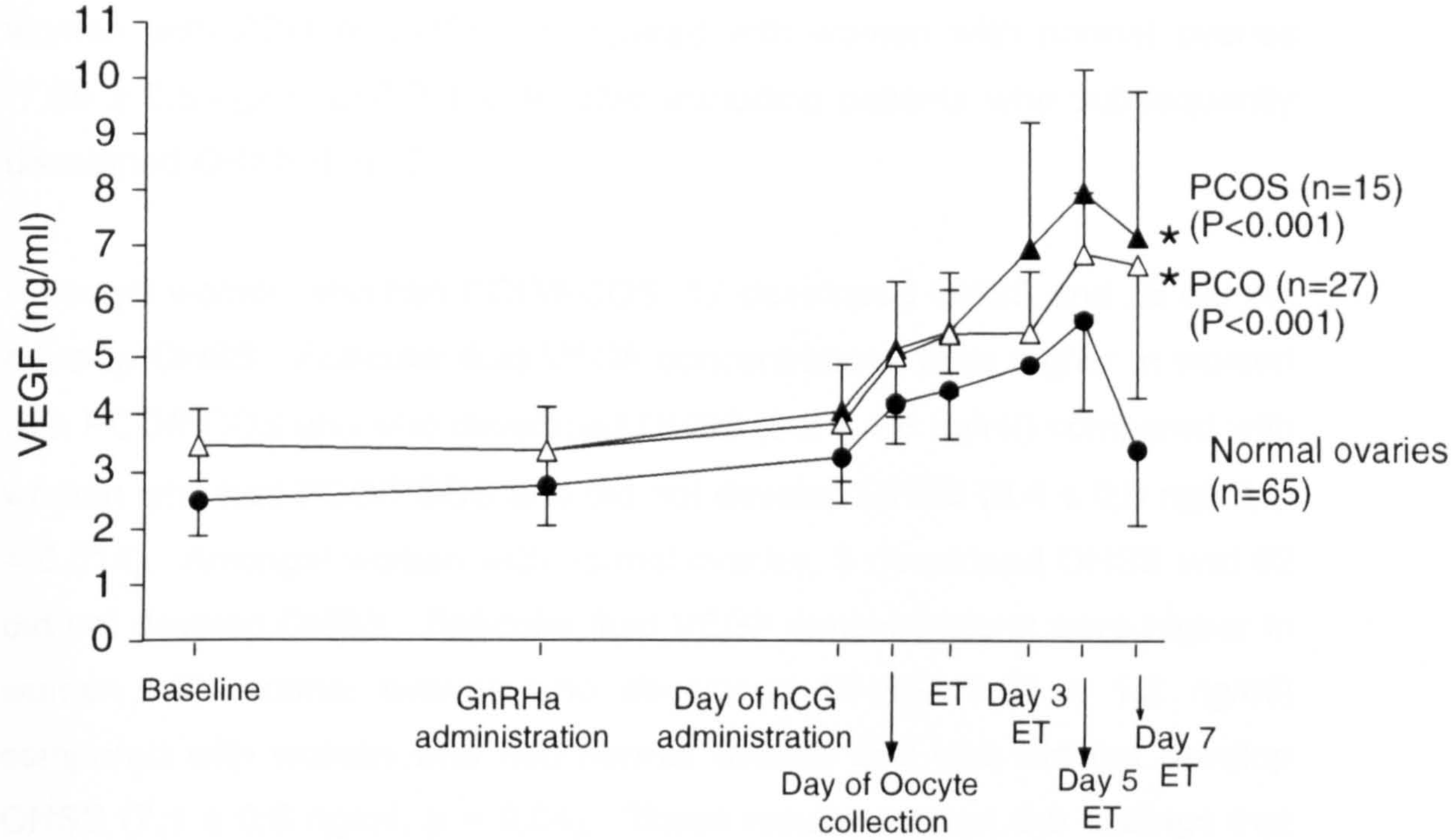


Figure 12.21

Serum VEGF concentrations during IVF cycles in women with normal ovaries (n = 65), PCO (n = 27) and PCOS (n = 15) (*p<0.001).

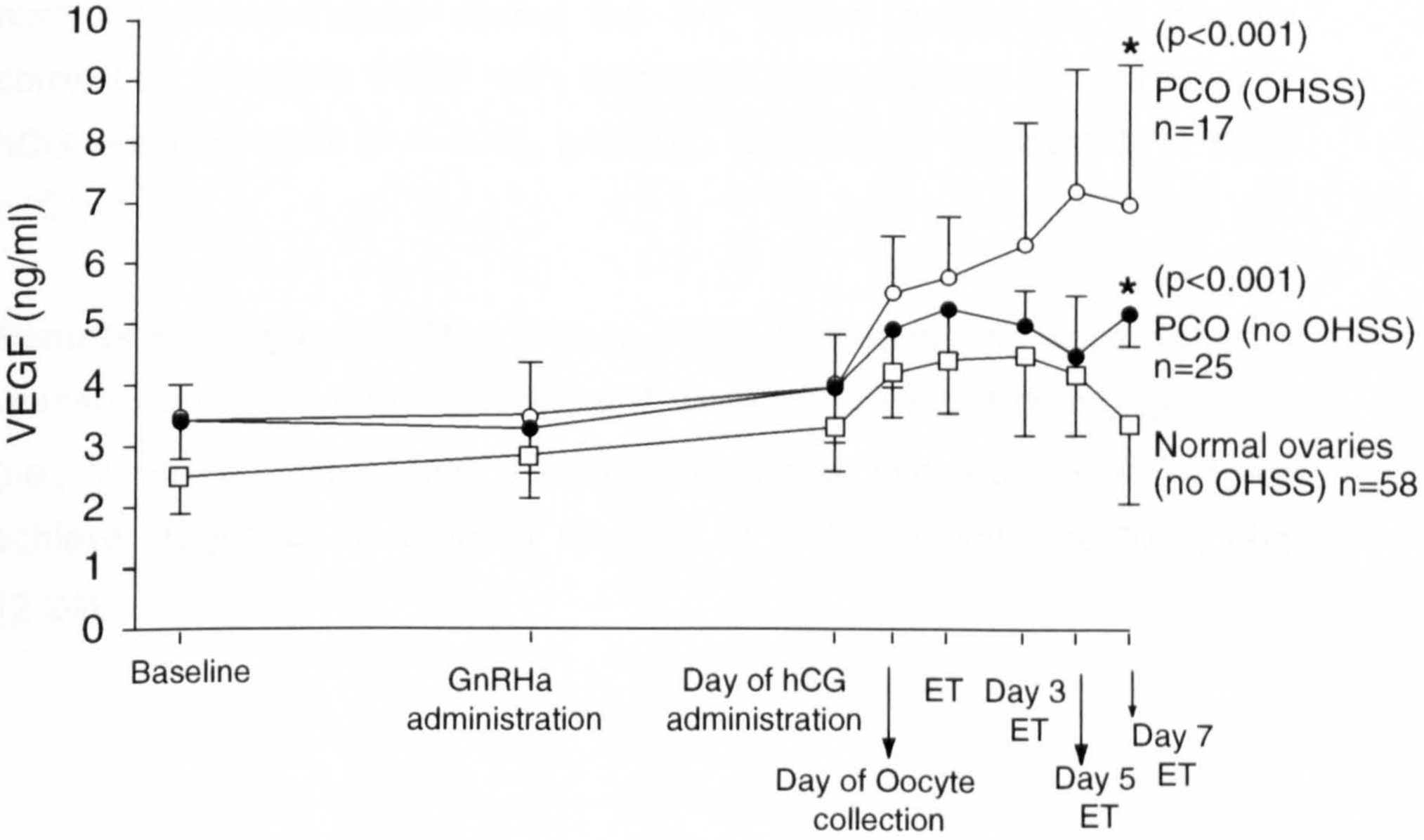


Figure 12.22

Serum VEGF concentrations in IVF cycles in women with normal ovaries and polycystic ovaries who developed and did not develop OHSS (*p<0.001).

Follicular fluid VEGF concentrations were higher (8.46 ± 0.6 ng/ml) in women with PCO and PCOS compared with women with normal ovaries (7.85 ± 0.5 ng/ml, $p<0.01$) even after excluding patients who subsequently developed OHSS (Fig 12. 23).

Amongst women who had PCO/PCOS, 17 developed OHSS and 25 did not develop OHSS. Follicular fluid VEGF concentrations were higher in women with PCO/PCOS and who developed OHSS (9.2 ± 1.6 ng/ml) compared with women who had PCO/PCOS and did not develop OHSS (8.4 ± 0.6 ng/ml, p = 0.014). Amongst women with normal ovaries, 3 developed OHSS and 62 did not develop OHSS. Follicular fluid VEGF concentrations were higher in women with normal ovaries who developed OHSS (8.23 ± 1.2 ng/ml) compared with women who had normal ovaries and who did not develop OHSS (7.1 ± 0.6 ng/ml, p = 0.04). These results support the findings that OHSS, independent of PCO/PCOS, contribute to significantly higher follicular fluid VEGF concentrations.

There were no correlations of serum VEGF concentrations with serum hormone concentrations during the IVF cycles except for a positive correlation of serum VEGF with oestradiol concentrations on the days of hCG administration (r = 0.48, p<0.001) and oocyte retrieval (r = 0.36, p<0.01).

Results in Pregnancy: Mean serum VEGF concentrations were higher in women who achieved pregnancy at 4 weeks (5.59 ± 0.8 ng/ml) gestation (i.e., 2 weeks post embryo transfer) compared with those who did not achieve pregnancy at a similar stage (3.11 ± 0.89 ng/ml, p<0.0001) (Fig 12.24).

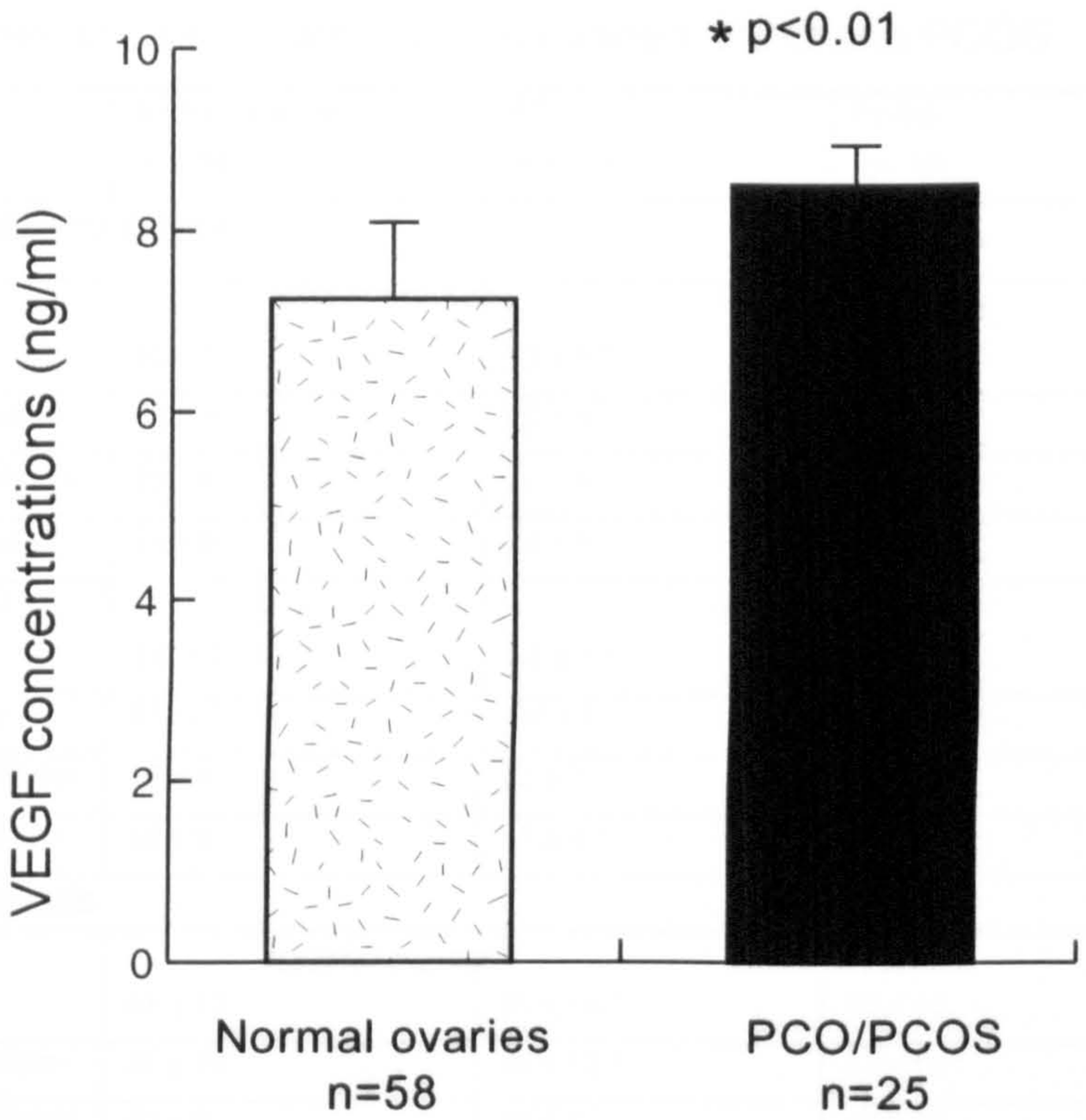


Figure 12.23

Follicular fluid VEGF concentrations in women with PCO and PCOS compared with women with normal ovaries (*p<0.01).

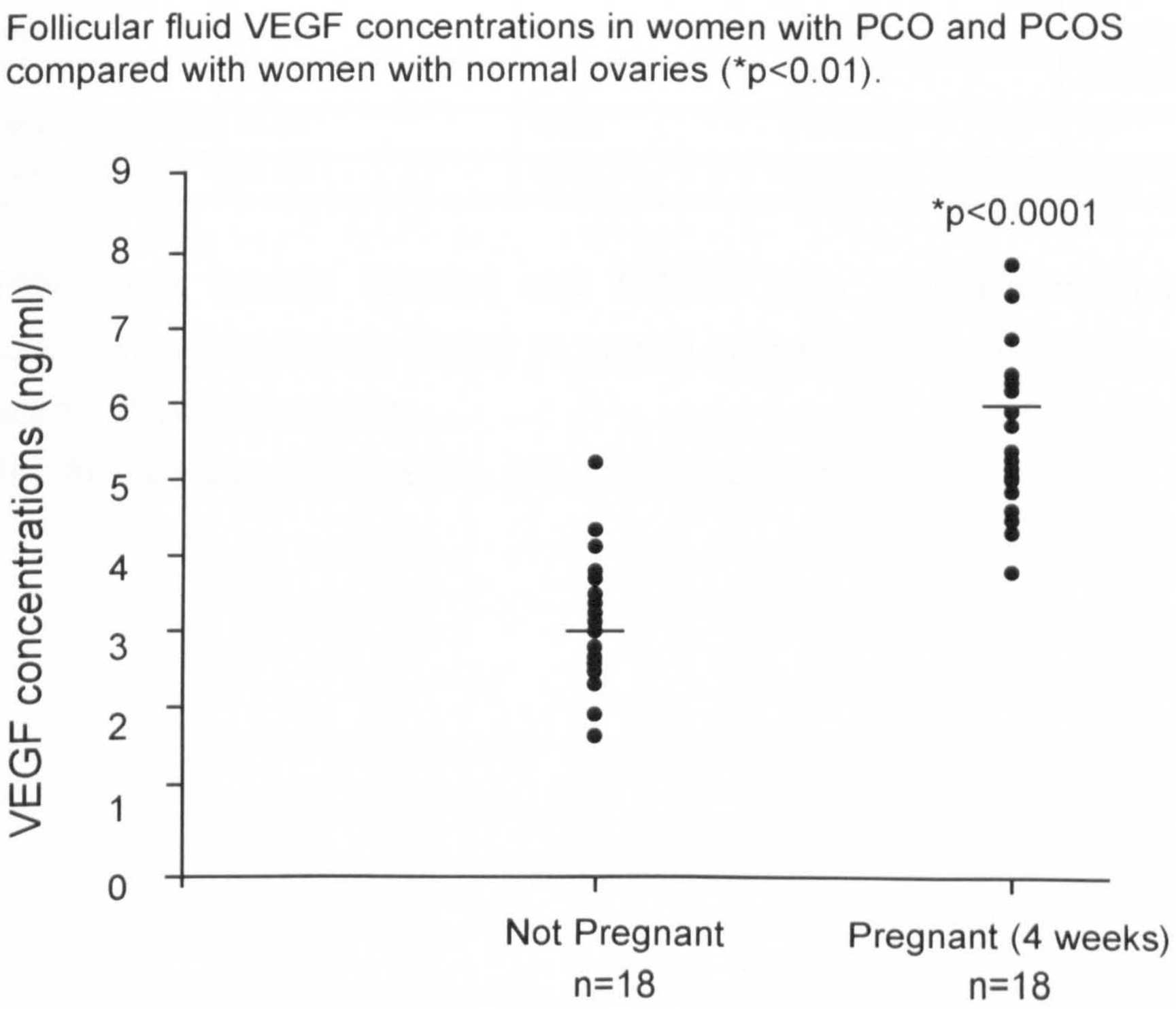


Figure 12.24

Serum VEGF concentrations in women who achieved pregnancy after IVF cycles at 4 weeks gestation (2 weeks post ET) (n=18) compared with those who did not achieve pregnancy (n=18) at a similar stage (*p<0.0001).

Table 12.5 Doppler blood flow velocities within ovarian stromal and uterine blood vessels in women with normal ovaries, PCO and PCOS.

Variable	Normal ovaries (n = 65)	PCO (n = 27)	PCOS (n= 15)	*P* value
Ovarian stromal blood vessels				
PSV (cm/sec) Baseline	10 ± 3	13 ± 3 *	15 ± 4 *	*P* < 0.0001
Day of hCG injection	14 ± 4	15 ± 5 *	20 ± 7 *	*P* < 0.003
Day of oocyte retrieval	23 ± 6	21 ± 4	20 ± 5	NS
Day embryo transfer	24 ± 9	22 ± 4	20 ± 1	NS
TAMXV (cm/sec) Baseline	6.9 ± 2	8.5 ± 1 *	11 ± 2 *	*P*< 0.0001
Pre hCG therapy	9.5 ± 3	9.8 ± 3	12 ± 5 *	*P* = 0.007
Day of oocyte retrieval	15 ± 4	13 ± 3	14 ± 2	NS
Day embryo transfer	16 ± 6	15 ± 4	14 ± 0.7	NS
Uterine blood vessels				
PSV (cm/sec) Baseline	41 ± 12	51 ± 16 *	39 ± 12	*P* = 0.01
Day of hCG injection	41 ± 12	46 ± 13 *	47 ± 14 *	*P* < 0.05
Day of oocyte retrieval	40 ± 8	48 ± 7	47 ± 12	NS
Day embryo transfer	43 ± 5	53 ± 8	49 ± 5	NS
TAMXV (cm/sec) Baseline	14 ± 6	17 ± 7 *	16 ± 4 *	*P* < 0.05
Pre hCG therapy	13 ± 4	15 ± 5 *	17 ± 6 *	*P* = 0.001
Day of oocyte retrieval	13 ± 4	16 ± 2	17 ± 0.8	NS
Day embryo transfer	15 ± 2	21 ± 6	19 ± 6	NS

* PSV (peak systolic velocity) and TAMXV (time averaged maximum velocity) were significantly higher in women with PCO and PCOS than in women with normal ovaries.

Note. All values are mean ± SD. NS = not significant.

Discussion:

Recent evidence has suggested a relationship between VEGF and ovarian perifollicular and stromal blood flow during ovarian stimulation cycles (Lee et al., 1997, Van Blerkom et al., 1997). In the present study, this relationship was explored in IVF treatment cycles. Changes in serum VEGF concentrations during an IVF treatment cycle are described for the first time in this study. Serum VEGF concentrations rose during the phase of ovarian stimulation and were significantly higher on the day of hCG administration. The concentrations rose further thereafter (Fig 12.18).

The results were consistent with the sequence of events, described in the vasculature and VEGF mRNA expression of the granulosa, theca and lutein cells prior to and after ovulation.

Changes in Doppler blood flow velocity and resistance indices within the ovarian stromal and uterine blood vessels during the IVF treatment cycles were similar to those previously described (Deutinger et al., 1989, Weiner et al., 1993). Doppler blood flow velocities correlated positively with serum VEGF concentrations. Van Blerkom and colleagues (1997) found perifollicular blood flow, as demonstrated by Doppler ultrasonography, correlated with serum VEGF concentrations.

Women who recruit an excessive number of follicles and produce multiple corpora lutea may overexpress VEGF, which, when it escapes from the ovary may be responsible for the fluid shift from the vascular bed to the extravascular space that characterises the syndrome of OHSS (Neulen et al., 1995).

Hence numerous studies have now described the presence of VEGF in the ascitic fluid (Mc Clure et al., 1994, Lee et al., 1997, Krasnow et al., 1996, Abramov et al, 1997, Agrawal et al., 1997), plasma (Abramov et al., 1997, Artini et al., 1998), serum (Krasnow et al., 1996, Lee et al., 1997, Ludwig et al., 1998, Moncayo et al., 1998), pleural fluid (Abramov et al., 1997, Agrawal et al., 1997), urine (Robertson et al., 1995) and follicular fluids (Amato et al.,

1996, Krasnow et al., 1996, Lee et al., 1997, Moncayo et al., 1998) of women undergoing ovarian stimulation for IVF and/or in those who subsequently developed OHSS. The study by McClure et al., 1994 also confirmed that ascitic fluid caused by liver failure had undetectable VEGF concentrations as measured by bioassay compared with ascitic fluid caused by OHSS.

The above studies have suggested that release of VEGF from the ovary may mediate the syndrome of ovarian hyperstimulation (OHSS). To study further the role of VEGF as a possible mediator of OHSS, differences were sought in serum and follicular fluid VEGF concentrations in women who developed OHSS and those who did not. Though VEGF concentrations rose during the phase of ovarian stimulation in all women, the rise was greater in women who subsequently developed OHSS compared with those who did not (Fig 12.19), consistent with the concept of VEGF as a mediator of OHSS. Further serum samples obtained three, five and seven days after embryo transfer in women who developed OHSS showed rising concentrations of VEGF compared with women who did not develop OHSS. In the latter the trend was of declining serum VEGF concentrations (Fig 12.19). These findings suggest that VEGF may provide an important nonsteroidal marker of the ovarian response to gonadotrophin stimulation.

These findings are consistent with those of Krasnow et al., 1996, Abramov et al., 1997, Lee et al., 1997, Ludwig et al., 1998, Moncayo et al., 1998 and Artini et al., 1998. Krasnow et al., 1996 did not however observe higher serum VEGF concentrations in women who developed OHSS until after 14 days of embryo transfer but their study did not include controls. Ludwig et al., 1998, observed that, although serum VEGF concentrations were elevated in women with severe OHSS, VEGF itself did not serve as satisfactory a clinical marker of the course of OHSS as the other traditional markers (haematocrit, fluid balance and body weight). They also observed that, although VEGF rose initially with the hCG stimulus, further rise of serum concentrations of hCG had no effect on VEGF concentrations. Artini et al., 1998, compared plasma VEGF concentrations in women at risk and

not at risk of developing OHSS. They observed that, in women at risk and who subsequently developed OHSS, plasma VEGF concentrations rose significantly after hCG administration. In women who were not at risk and who did not develop OHSS, plasma VEGF concentrations declined or remained steady after hCG administration.

Follicular fluid VEGF concentrations were higher in women who developed OHSS compared with women who did not. Follicular fluid concentrations were found to be 3 times higher than serum VEGF concentrations, in contrast to results by Krasnow et al., 1996 who found a 100 fold higher concentration of VEGF in follicular fluid and Artini et al., 1998 who found follicular fluid VEGF concentrations to be 10 times higher compared with plasma VEGF. Lee et al., 1997 observed a 10 fold higher concentration of VEGF in the follicular fluid of women who developed OHSS compared with serum. A study by Moncayo et al., 1998 too, confirmed follicular fluid VEGF concentrations to be higher than serum VEGF concentrations. Lee et al., 1997 and Moncayo et al., 1998 observed a positive correlation between follicular fluid VEGF and progesterone concentrations. This relationship was not studied in this thesis.

The causes of these differences are uncertain but may in part at least be explained by differing assays and differing methods of sample storage.

Using a bioassay, McClure et al., 1994, described high levels of VEGF in the peritoneal fluid of 3 patients with OHSS. They found that 70% of the capillary permeability activity in OHSS ascites was neutralised by antiserum to human recombinant VEGF. Increased concentrations of VEGF were observed in the peritoneal fluid in women who developed OHSS in an earlier study (Agrawal et al., 1997) (Fig 12.25).

Changes in ovarian stromal and uterine artery Doppler blood flow velocities (PSV and TAMXV) corresponded to changes in serum VEGF (Fig 12.18). Further studies are required to explore the links between circulating VEGF and changes in Doppler blood flow velocities. Resistance indices (PI and RI) within ovarian stromal blood vessels and uterine arteries were not

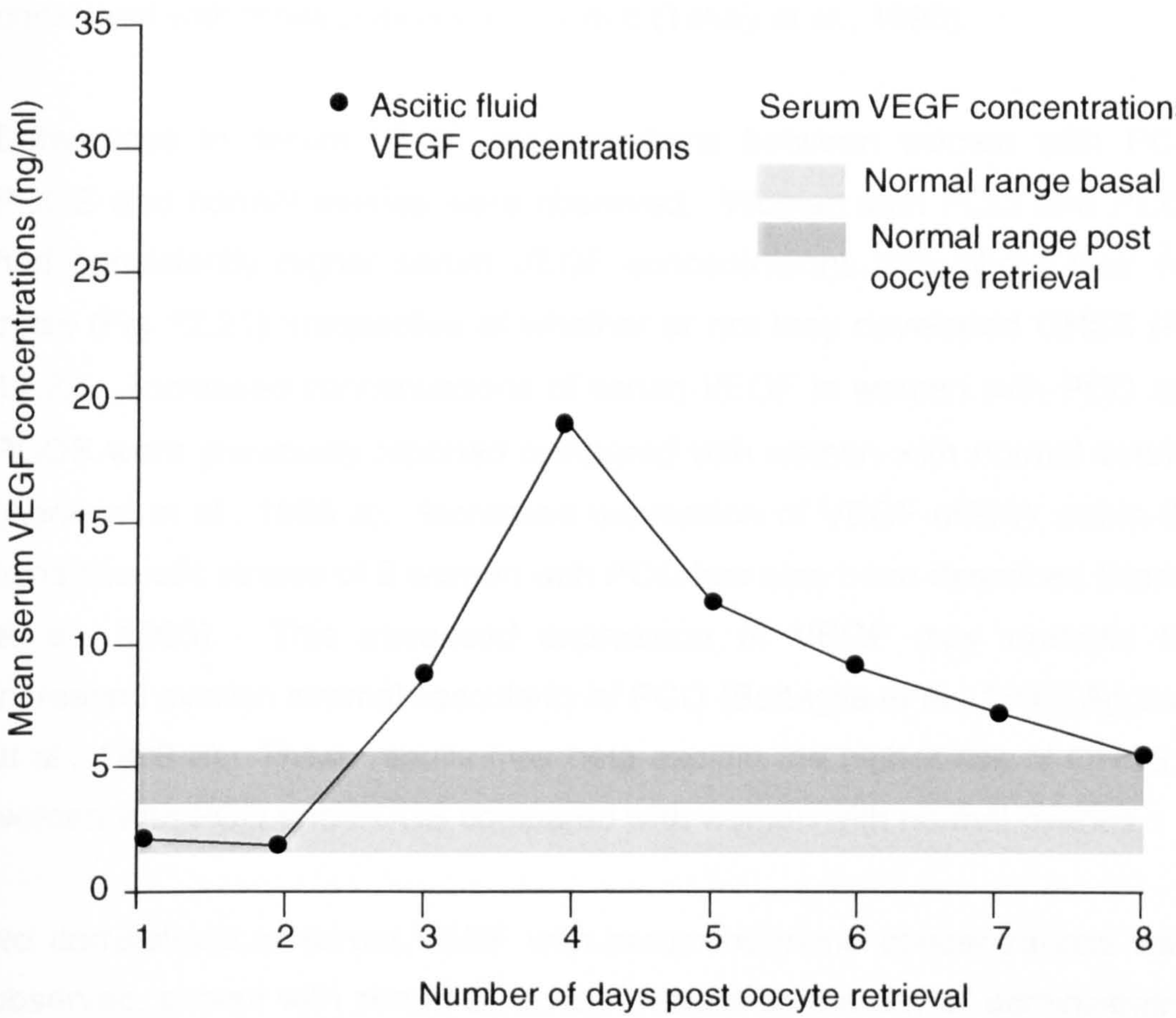


Figure 12.25

Serum and ascitic fluid VEGF concentrations after ooctye retrieval in a woman who developed severe OHSS.

significantly different in the two groups of women. These results are consistent with those previously reported (Tekay et al., 1995).

Differences in serum VEGF concentrations between women with PCO, PCOS and normal ovaries were observed. Women with PCO and PCOS had consistently higher serum VEGF concentrations throughout their IVF cycle (Fig 12.21) irrespective of whether or not they developed OHSS (Fig 12.22). Increased concentrations of serum VEGF in women with PCO and PCOS were previously reported compared with women with normal ovaries (Agrawal et al., 1998 a). Increased expression of VEGF mRNA within the hyperthecotic stroma of 3 women with PCO has also been described (Kamat et al., 1995). This increased expression of VEGF may underlie the increased ovarian stromal vascularity of PCO (Battaglia et al., 1995, Agrawal et al., 1998 a). These results may help explain the higher risk of OHSS in women with PCO and PCOS compared with women with normal ovaries.

No correlations of serum VEGF with serum hormone concentrations were observed, except with serum E_2 concentrations on the day of administration of hCG and on the day of egg collection. Further larger studies are required to explore this correlation. This result is different from our earlier observations during the normal menstrual cycle on 12 patients. A positive correlation between serum VEGF concentrations and oestradiol and between VEGF and testosterone concentrations in the early follicular phase of the menstrual cycle was observed.

VEGF concentrations were higher in women who achieved pregnancy after IVF treatment at 4 weeks of gestation compared with women who did not achieve pregnancy. This could be explained by the persistence of the corpus luteum, placentation and early embryogenesis, which are known to be VEGF dependent (Ferrara and Davis-Symth, 1997). This result is similar to that reported by Lee et al., 1997 who observed higher concentrations of VEGF in sera of women who achieved pregnancy than those who did not achieve a pregnancy. Evans et al., 1997 and Wheeler et al., 1999 also confirmed serum VEGF concentrations to be elevated during the first 10

weeks of pregnancy.

To support these clinical observations, in - vitro studies have demonstrated that decidual transformation leads to upregulation of VEGF expression in decidual cells (Jakeman et al., 1993, Shweiki et al., 1993, Chakraborty et al., 1995). Within the implantation site, high steady state levels of VEGF mRNA are expressed in the extra-embryonic giant trophoblast cells. VEGF thus produced forms a gradient of angiogenic activity, directing the growth and migration of endothelial cells towards the embryo. It was also observed that steady state levels of VEGF mRNA and VEGF receptors were highest during the implantation period and the transcripts occupied an endometrial apical epithelial position around the implantation site (Shifren et al., 1996). The epithelial distribution suggests that VEGF may be secreted into the gland lumina, potentially influencing the nutrition and apposition of the developing blastocyst (Hornung et al., 1998). It was also suggested that the rapid growth of the placental blood vessels throughout pregnancy may be related to hypoxia induced angiogenesis mediated via VEGF (Wheeler et al., 1995).

VEGF also plays a role in embryonic development. VEGF mRNA is expressed in several organs of the mouse and rat embryo, such as the heart, vertebral column, brain and the kidney (Jakeman et al., 1993, Breier et al., 1992). In the human fetus (16 -22 weeks), VEGF mRNA expression is detectable in virtually all tissues, including the liver, kidney and spleen (Shifren et al., 1994). Immunocytochemical analysis has shown the presence of VEGF protein principally in embryonic epithelial cells, myocytes and the smooth muscle cells lining the blood vessels but not in vascular endothelial cells (Shifren et al., 1994). These findings support that paracrine mechanism of action of VEGF whereby it is secreted by nonendothelial cells and modulates the activities of the adjacent vascular endothelium.

Study 4: Study of serum vascular endothelial growth factor (VEGF) in in-vitro fertilisation cycles as a predictor of the risk of ovarian hyperstimulation syndrome.

There were no significant differences between women with and without OHSS with respect to age, duration of infertility (DOI), parity, previous IVF attempts, the treatment received (IVF or ICSI), the infertility diagnosis, and the basal serum FSH, LH and E_2 concentrations. Basal serum testosterone concentrations were higher (1.54 ± 1.1 nmol/l vs. 0.87 ± 0.5 nmol/l, $p<0.05$) in women who developed OHSS, probably because of the higher proportion of women with PCOS in this group of women. Thus women who developed OHSS were more likely to have PCO and PCOS (85%) than those who did not develop OHSS (29.8%, $p <0.005$) (Table 12.6).

Mean serum E_2 concentrations on the day of administration of hCG in women who received gonadotrophins containing FSH alone were lower (5692 ± 2735 pmol/l) than in women who received HMG (8006 ± 5860 pmol/l, $p<0.01$). These differences are not accounted for in the risk prediction of the development of OHSS since there was no difference between these two groups (FSH or HMG for ovarian stimulation) in the proportion of patients who developed OHSS.

The mean serum E_2 concentrations, mean follicle numbers on the day of hCG administration and the mean number of oocytes retrieved are shown in Table 12.6.

Table 12.6: Variables used for risk prediction of ovarian hyperstimulation syndrome.

	Study group			
	A	B	C	
Variables	Patients without OHSS (n = 87)	Patients with OHSS (n =20)	Patients with Moderate/severe OHSS (n = 10)	*P* value
Serum E_2 levels (pmol/L)	5759 ± 3285	9762 ± 5844 *	12525.7 ± 7141**	*P*<.001
No. of Follicles	15.9 ± 9.4	29.2 ± 8.9 *	33.1 ± 6.7 **	*P*<.01
No. of Oocytes obtained	10.6 ± 5.2	21.6 ± 7.8 *	23.6 ± 7.5 **	*P*<.005
No. of patients with PCO (%)	26 (29.8)	17 (85) *	10 (100) **	*P*<.01
Serum "VEGF rise" (ng/mL)	0.87 ± 0.3	1.56 ± 0.5 *	1.58 ± 0.6 **	*P*<.0001
Serum VEGF levels on day of oocyte collection (ng/mL)	4.42 ± 0.8	5.37 ± 0.9 *	5.68 ± 1.1 **	*P*<.01

* Values statistically significantly higher in Group B compared with Group A

* * Values statistically significantly higher in Group C compared with Group A

Note: All values are means ± SD unless otherwise indicated.

PCO - Polycystic ovaries; OHSS - Ovarian hyperstimulation syndrome.

The sensitivity, specificity and the positive predictive values of each of the variables as a marker of OHSS are shown in Table 12.7.

Mean serum VEGF concentrations rose significantly in all women during the phase of ovarian stimulation and was higher on the day of hCG administration (3.59 ± 0.91 ng/ml) compared with early follicular phase i.e., at the beginning of the IVF treatment (2.93 ± 0.77 ng/ml, p <0.0001). Serum VEGF rose further after hCG administration to 4.59 ± 0.93 ng/ml on the day of oocyte retrieval and to 4.88 ± 1.02 ng/ml on the day of embryo transfer. Women who developed OHSS had consistently higher serum VEGF concentrations before and throughout their IVF cycle except after pituitary desensitisation (Fig 12.19). The rise of serum VEGF concentrations from the day of administration of hCG to the day of egg collection (subsequently referred to as "VEGF rise") proved to be a good marker of the development of OHSS (Table 12.7). "VEGF rise" was higher in women who developed OHSS compared with women who did not develop OHSS (Table 12.6).

Though serum VEGF concentrations were higher in women who developed OHSS throughout the IVF cycle, including the early follicular phase (prior to starting IVF treatment), on the days of hCG administration, egg collection and embryo transfer, serum VEGF concentrations on these days as single markers did not predict the development of OHSS (statistical analysis not shown).

Sensitivity, specificity and predictive values of various markers and a combination of markers showed that the "VEGF rise" along with follicle numbers on the day of hCG administration and a pre - treatment diagnosis of PCO were the most reliable markers of OHSS (Table 12.7). The combination of these 3 factors gave a positive predictive value of 84.2% for all OHSS cases (sensitivity 85%, specificity 96.5.7%) and of 52.6% for moderate and severe OHSS cases (sensitivity 100%, specificity 90.7%). A combination of "VEGF rise" along with follicle numbers on the day of hCG administration also predicted OHSS effectively (Table 12.7).

Table 12.7: Sensitivity, Specificity and predictive values of criteria used for risk estimation of ovarian hyperstimulation syndrome.

Variables	All cases of OHSS (n = 20)			Moderate/severe OHSS (n =10)		
	Sensitivity (%)	Specificity (%)	PPV (%)	Sensitivity (%)	Specificity (%)	PPV (%)
Serum E_2 levels	75	49	25	90	52	15
No. of follicles	95	52	31	100	56	19
No. of oocytes	80	79.3	47	90	74.2	26.4
Presence of PCO	85	70	40	100	66	23.2
VEGF rise	100	60	40	100	60	40.3
VEGF rise + follicles	90	93	75.2	100	85.5	43.4
VEGF rise + follicles + PCO	85	96.5	84.2	100	90.7	52.6
VEGF rise + follicles + PCO + E_2	75	97.7	88	90	93.8	60
VEGF rise + oocytes	80	91.9	66.6	90	85.5	39.1
E_2 + follicles + VEGF rise	70	94.2	66.6	90	90	50
E_2 + follicles + PCO	92	87	54	90	85	38

Note: PCO = Polycystic ovaries; VEGF = Vascular endothelial growth factor. Cut - off values for variables were established by receiver operating curves and were as follows: 6,500 pmol/l for serum E_2 concentrations; 25 for follicle number; 15, for number of oocytes retrieved; and 1 ng/mL for "VEGF rise".

A likelihood ratio was calculated, which compared the log likelihood of 2 logistic regression models, one which (model 1, Table 12.8) included all 4 continuous variables (i.e., follicle number, log transformed E_2 concentration, presence of PCO and "VEGF rise") and the other of which excluded the "VEGF rise". The result showed that adding "VEGF rise" (model 2, Table 12.8) as a continuous variable significantly improved the model for calculating the odds of developing moderate and severe OHSS ($p = 0.015$) (Table 12.8). Similarly adding serum VEGF concentrations on the day of egg collection as a continuous variable significantly improved model 2 ($p = 0.02$). The odds ratios and confidence intervals for the various markers are shown in Table 12.8.

Table 12.8: Odds ratio and confidence interval for various variables that serve as markers of ovarian response.

	Marker	Odds ratio	95% confidence intervals
Model 1	Log E_2 levels	5.32	1.1 - 24.6
	No. of follicles	1.08	1 -1.1
	Presence of PCO	5.75	0.67 - 49
	VEGF rise	11.3	1.4 - 90.4
Model 2	Log E_2 levels	4.8	1.1 - 20.2
	No. of follicles	1.1	1.02 - 1.19
	Presence of PCO	2.57	0.35 - 18.4
	VEGF levels on day of oocyte collection	3.01	1.01 - 8.9

Note: All values were determined by regression analysis and by the likelihood ratio test.

Within the ovarian stromal blood vessels, Doppler blood flow velocities (PSV and TAMXV) were higher in women who developed OHSS compared with women who did not (Fig 12.19). There were however no differences between the PI and RI within the ovarian stromal blood vessels and no differences in blood flow velocities within the uterine arteries between the two groups of women. Though ovarian stromal blood flow velocities were higher in women who developed OHSS compared with women who did not, probably because of a higher number of patients with PCO in the group who developed OHSS, blood flow velocities on the day of hCG administration did not predict the risk of developing OHSS (statistics not shown).

Discussion:

Prediction of OHSS has traditionally focused on indexing the endocrine and ultrasound response to ovarian stimulation. Numerous reports attest to the value of oestradiol (E_2) measurements in blood or urine and based on such measurements, criteria have been developed for withholding hCG in protocols of ovarian stimulation (Brinsden et al., 1995). While it has been known that high concentrations of E_2 are an immediate precursor of the syndrome, oestradiol itself does not cause OHSS (Meirow et al., 1996). For instance a patient with partial 17-20 desmolase deficiency has developed severe OHSS despite low serum E_2 concentrations. Moreover elevated serum E_2 concentrations predict OHSS in only a quarter of cases.

Other criteria for the prediction of OHSS, such as number of follicles on the day of hCG administration, the presence of polycystic ovaries on ultrasonography and number of oocytes retrieved, may be subject to inter - observer variations during ultrasonography and inter - operator differences during oocyte retrieval (Asch et al., 1991, Delvigne et al., 1993). As individual markers of OHSS, both increased follicle and oocyte numbers predict only a quarter of the cases of OHSS (Rizk et al., 1991).

Although the ovarian prorenin - renin - angiotensin system (Ong et al., 1991) and ovarian cytokines (Revel et al., 1996) may be involved in the pathogenesis of OHSS, currently available measurements of these systems

do not predict the risk of developing OHSS (Navot et al., 1987).

The study of Mc Clure et al, 1994 using a bioassay of VEGF suggested that release of VEGF mediated OHSS. The relationship of VEGF to OHSS has been confirmed using immunological assays by work in our own (Agrawal et al., 1997) and other laboratories (Lee et al., 1997). VEGF therefore, provides a potentially useful, non-steroidal index of the ovarian response to gonadotrophin stimulation.

Since the ovary produces maximum amounts of VEGF after ovulation or after hCG administration (Neulen et al., 1995), serum VEGF concentrations prior to hCG administration would not be expected to predict the risk of OHSS. Although serum VEGF concentrations were higher in women who developed OHSS compared with women who did not, serum VEGF on the day of hCG administration or earlier did not predict the risk of developing OHSS. The results show however, that the rise of serum VEGF concentrations from the day of hCG administration until the day of egg collection ("VEGF rise") may provide an important nonsteroidal marker of OHSS.

In this study, "VEGF rise" predicted 40 % of all cases of OHSS and 40.3 % of moderate and severe cases of OHSS, with no false negative results. However serum VEGF concentrations as single markers during early follicular phase, on the day of hCG administration, on the day of egg collection or embryo transfer, did not predict the development of OHSS.

These prediction rates for all cases of OHSS and for moderate and severe OHSS were only marginally better than serum E_2 on the day of hCG administration (Table 12.7). There were however no false negative results with "VEGF rise". It is perhaps more important to have lower false negative results than increase the predictive rates, since IVF treatment is never a vital necessity but OHSS may be life threatening (Delvigne et al., 1993b).

The diagnosis of polycystic ovaries, as indexed on ultrasonography by the

'necklace pattern of ovaries' (Delvigne et al., 1991, Rizk et al., 1992, MacDougall et al., 1993) remains a crucial pre - stimulation marker of OHSS, since OHSS occurs more commonly in women with PCO (Delvigne et al., 1991). Using immunohistochemistry, Kamat et al., 1995 described increased expression of VEGF mRNA in the theca cells in 3 polycystic ovaries. Work described here has indicated that women with polycystic ovaries have higher basal (pre - stimulation) serum concentrations of VEGF than women with normal ovaries on ultrasound (Agrawal et al., 1998 a). In the present study, higher serum VEGF concentrations were observed at all stages of ovarian stimulation in women with polycystic ovaries compared with those with normal ovaries. Subjects who went on to develop OHSS (85% of whom had PCO) had higher VEGF concentrations compared with those who did not develop OHSS (29% of whom had PCO).

In the present study shown and as reported elsewhere (Agrawal et al., 1998 c), the mean serum E_2 concentrations in patients receiving FSH preparations were significantly lower than in women who received HMG during IVF using the "long protocol" of GnRH analogues. Yet there were no differences in the risk of OHSS in relation to the type of gonadotrophins used. Further, using elevated serum E_2 concentrations on the day of hCG administration as a marker of OHSS, OHSS could be predicted in only 25% of cases, similar to results published previously (Forman et al., 1990, Morris et al., 1995). A clear cut-off value for serum E_2 concentration, above which OHSS is likely to occur, has not been established, largely because of differences in IVF techniques and variability in E_2 assays. Thus the particular serum E_2 concentration on the day of hCG administration as a criterion for withholding hCG injection has varied from 800 pg/ml (2938 pmol/l) to 6000 pg/ml (22026 pmol/l) (conversion factor to S.I units 3.671) (Forman et al., 1990, Asch et al., 1991, Rizk et al., 1992, Delvigne et al., 1993, a, b). Further, one would expect lower E_2 concentrations as schedules of ovarian stimulation shift from combined gonadotrophins to preparations containing FSH alone (Agrawal et al., 1998 c) making the use of E_2 as a marker of OHSS increasingly unreliable.

Using the number of follicles observed on the day of hCG administration or the number of oocytes retrieved is also subject to variations because of differences in ultrasound equipment and in operator techniques for oocyte retrieval (Asch et al., 1991). Previous reports have shown that increased follicle numbers on the day of hCG administration predicts OHSS in only a quarter of the cases (Forman et al., 1990). Using the same (number of follicles recruited) criterion, only 31% of all OHSS cases could be predicted and 19% of moderate and severe cases of OHSS. Once again, there are no agreed cut-off levels for follicle numbers (range 10 - 35) or oocytes retrieved (range 14 - 30) above which the risk of developing OHSS has been shown to be increased (Forman et al., 1990, Asch et al., 1991, Delvigne et al., 1993, a, b)

Though several authors have recommended the use of ultrasonography and serum E_2 concentrations on the day of hCG administration for predicting the risk of OHSS (Rizk et al., 1992), it was observed that a combination of pre treatment diagnosis of polycystic ovaries together with follicle numbers on the day of hCG administration and "VEGF rise" gave the highest prediction rates for the risk of developing OHSS (Table 12.8). Addition of serum E_2 on the day of hCG administration improved these rates marginally, although false negative rates then increased from 15% to 25% for all cases of OHSS and from 0 to 10% for moderate and severe OHSS cases (Table 12.8). Further, a likelihood ratio test showed that adding "VEGF rise" or serum VEGF on the day of egg collection as a continuous variable to follicle number, serum E_2 concentrations on the day of hCG administration and the presence of PCO during a regression analysis, made a significant contribution to the model in predicting OHSS. However, no single criterion predicted the risk of developing OHSS reliably. Delvigne and colleagues (1993, a, b) have also reported the lack of predictive power of individual criteria and they suggested a step-wise discriminant analysis of various variables (Log serum E_2 rise, HMG dose, oocytes obtained, LH/FSH ratio, necklace sign of the ovaries) to provide a predictive mathematical function for evaluating the risk of OHSS prior to and after hCG administration. They obtained prediction rates of 76.1% and 78.5% with a mean of 18.1% false

negatives for all OHSS cases. This result was superior to an evaluation based on E_2 and/or follicle number or oocytes obtained. It seems therefore that serum VEGF measurements on the day of hCG administration and on the day of egg collection, together with a count of total follicles on the day of hCG administration and a pre-treatment diagnosis of PCO, may have advantage over serum oestradiol concentrations in terms of the prediction of OHSS. Such a conclusion would of course need to be concluded in a prospective study.

Study 5: Effect of menopause and HRT on serum VEGF concentrations

There were no statistically significant differences in the age (63.3 ± 7.8 vs. 64 ± 9.3 years), body mass index (BMI) (24.7 ± 4.1 vs. 25.4 ± 3.7 kg/m^2), duration of menopausal therapy (7.8 ± 3.5 vs. 6.8 ± 2.3 years), prevalence of smoking, alcohol consumption or hypertension between women who had had a hysterectomy compared with those who had not had a hysterectomy. Similarly there were no statistically significant differences in these parameters between the various treatment groups (Table 12.9 and 12.10).

Table 12.9: Characteristics of women with previous hysterectomy on different menopausal therapy

	Group 1 (no HRT) n = 6	Group 2 (Tibolone) n = 20	Group 3 (TRDE) n = 25	Group 4 (CEO) n = 16	*P* value
Age (years)	63 ± 9.6	65.6 ± 9.8	62.1 ± 9.2	61.9 ± 9	NS
BMI (kg/m^2)	24.8 ± 3.7	24.3 ± 3.4	25.1 ± 4.2	24.7 ± 3.2	NS
Duration of HRT (years)	---	6.2 ± 1	8.12 ± 2.7	9.2 ± 3	NS

TRDE - Transdermal oestradiol

CEO - Conjugated equine oestrogens

HRT - Hormone replacement therapy

* All values are mean ± SD

Table 12.10: Characteristics of women with uterus in situ on different menopausal therapies

	Group 1 (no HRT) n = 34	Group 2 (Tibolone) n = 56	Group 3 (TRDE) n = 24	Group 4 (CEO) n = 18	*P* value
Age (years)	65.3 ± 8	66.2 ± 8.1	64.3 ± 8	64.2 ± 8.1	NS
BMI (kg/m^2)	26.0 ± 4.4	25.3 ± 3.5	25.7 ± 3.2	23.7 ± 4.1	NS
Duration of HRT (years)	---	6.6 ± 1.34	7.31 ± 2.3	7.8 ± 2.6	NS

TRDE - Transdermal oestradiol

CEO - Conjugated equine oestrogens

HRT - Hormone replacement therapy

* All values are mean ± SD

Healthy postmenopausal women who did not receive menopausal therapy and who had their uterus in situ had a significantly higher mean VEGF concentration (4.15 ± 2.7 ng/ml) than those who had had a previous hysterectomy (2.62 ± 2.1 ng/ml, $p < 0.05$) (Fig 12.26).

The mean serum VEGF concentration in women on menopausal therapy was higher (4.45 ± 2.4 ng/ml) than that of women who were not on HRT (3.09 ± 2 ng/ml, $p = 0.006$) irrespective of whether they had a previous hysterectomy. When all women were considered, differences in mean serum VEGF concentrations between women on different HRT preparations were not significantly different.

However, women who had their uterus in situ, and who were therefore receiving treatment with oestrogens combined with progestogens, had lower VEGF concentrations compared with those who had a hysterectomy who were receiving oestrogens alone.

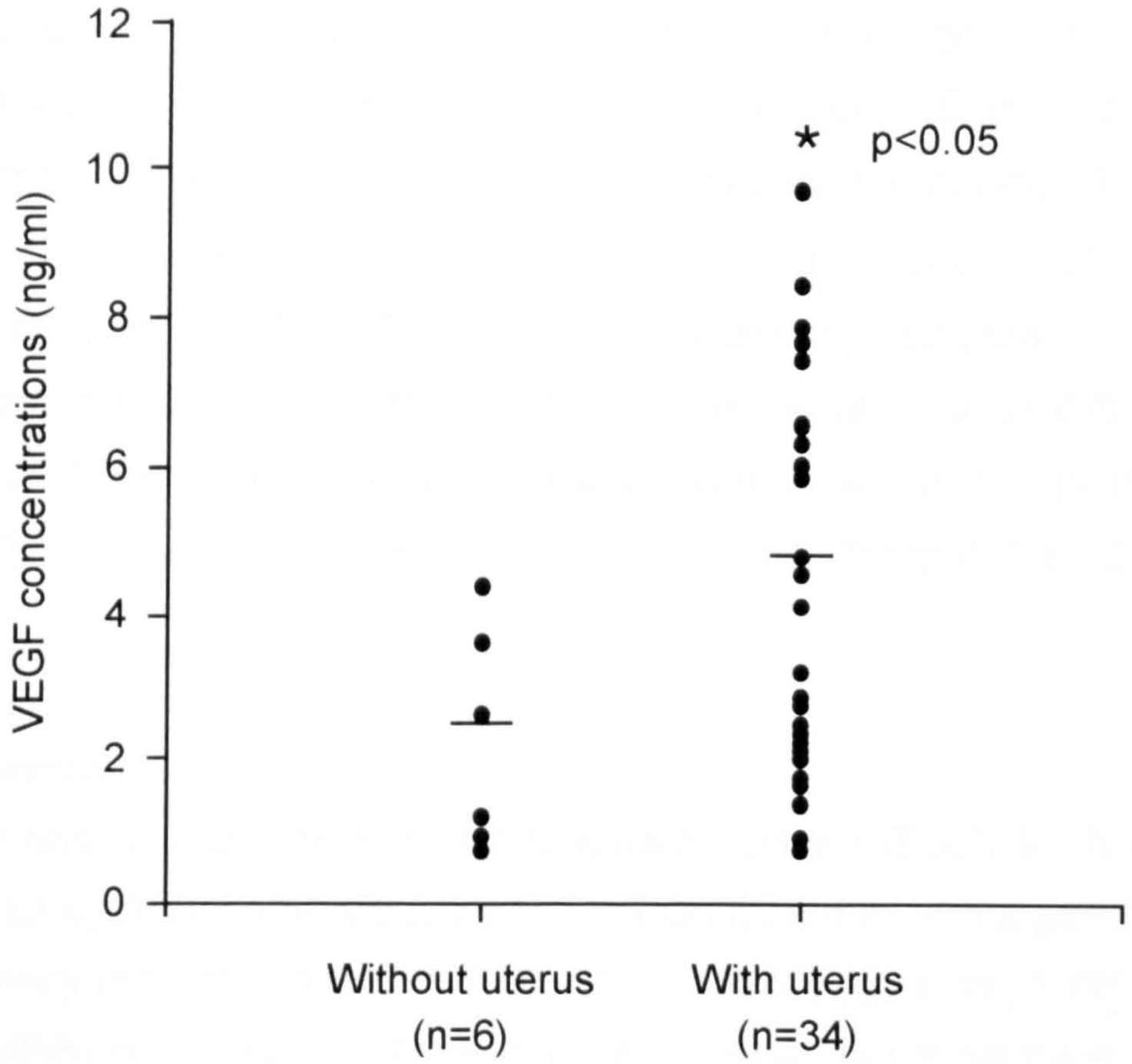


Figure 12.26

Serum VEGF concentrations in postmenopausal women not receiving HRT with previous hysterectomy (n = 6) or uterus in situ (n = 34)(*p<0.05).

In a subgroup analysis of the postmenopausal women who had had a hysterectomy, mean serum VEGF concentrations were significantly higher in those receiving conjugated equine oestrogens alone (5.88 ± 2.2 ng/ml) compared with women who were not on menopausal therapy (2.62 ± 2.1 ng/ml), on tibolone (4.21 ± 2.4 ng/ml) or on transdermal oestradiol (4.27 ± 2.1 ng/ml) (p = 0.02), (Fig 12.27). In contrast, subgroup analysis of postmenopausal women with uterus in situ demonstrated no difference in serum VEGF concentrations among the different treatment groups (Group 1, 4.15 ± 2.7, Group 2, 4.21 ± 3.1, Group 3, 3.7 ± 2.7, Group 4, 5.1 ± 2.37) (Fig 12.28).

Discussion:

Fluctuations of vascular endothelial growth factor (VEGF) in the normal menstrual cycle have previously been demonstrated in histological sections of the ovary (Kamat et al., 1995. Koos et al., 1995, Torry et al., 1996, Ferrara et al., 1998) and in serum (Agrawal et al., 1999 a), but there have been no reports of serum VEGF concentrations in postmenopausal women, on or off hormone replacement therapy.

Evidence that a major source of circulating VEGF in women is the reproductive tract comes from several observations (Kamat et al., 1995, Koos et al., 1995, Torry et al., 1996, Ferrara et al., 1998, Agrawal et al., 1999 a). VEGF mRNA is present in theca and luteinised granulosa cells and serum VEGF rises in the luteal phase of the menstrual cycle. Lastly, over production of VEGF may play a role in the pathogenesis of ovarian hyperstimulation syndrome (Mc Clure et al., 1994, Agrawal et al., 1998 a, 1999 b). Since cyclical fluctuations of serum VEGF concentrations occur in the normal ovulatory cycle (Agrawal et al., 1999 a), it was hypothesised that serum VEGF concentrations would fall after the menopause because of the loss of ovarian function. Further, as the uterus is one source of VEGF production (Torry et al., 1996), it was also considered that hysterectomy might also lower serum VEGF concentrations.

In the human ovulation cycle, cyclical fluctuations in uterine and ovarian Doppler blood flow velocities have previously been demonstrated, which

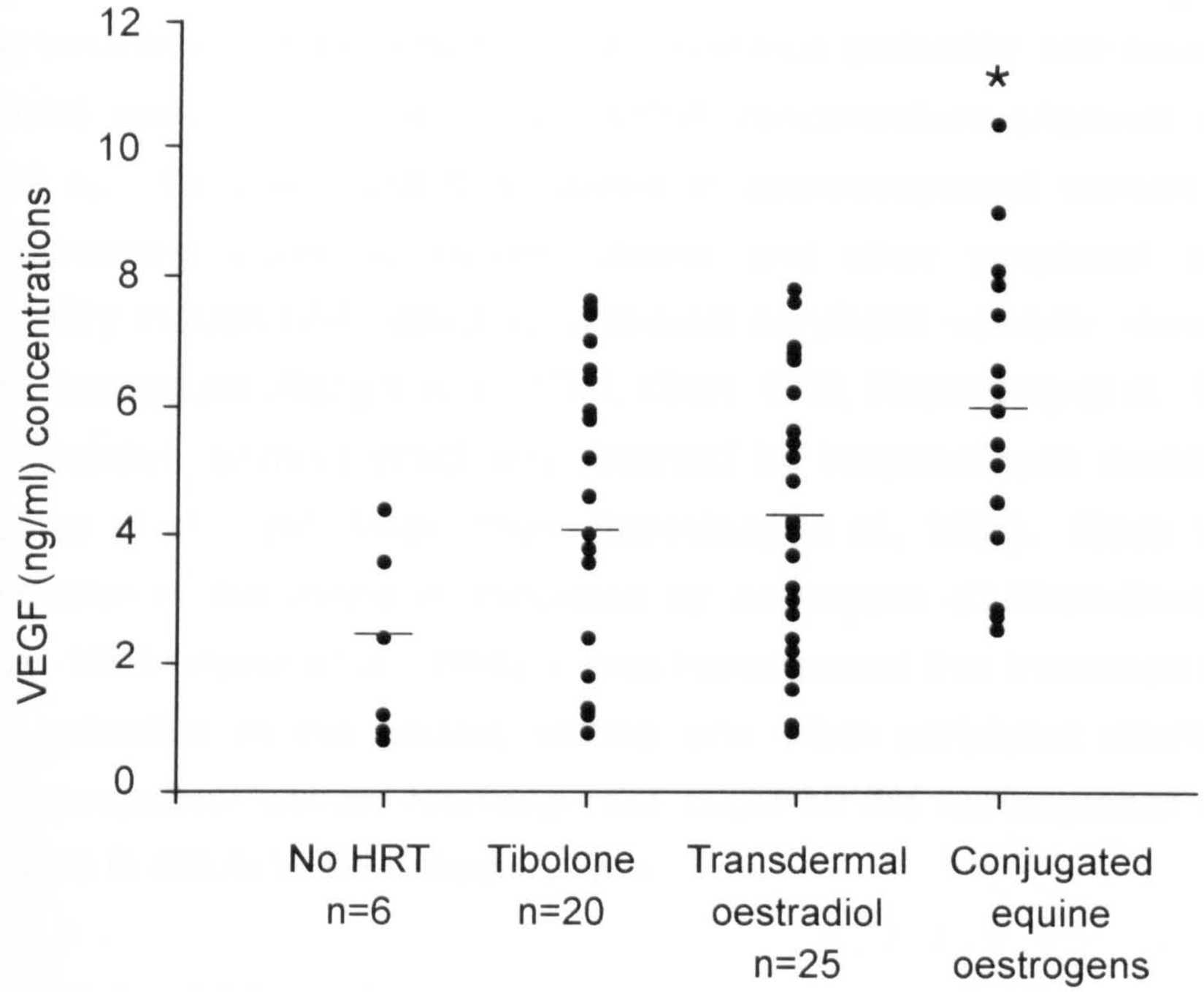


Figure 12.27

Serum VEGF concentrations in postmenopausal women with previous hysterectomy receiving different forms of HRT (*p=0.02)

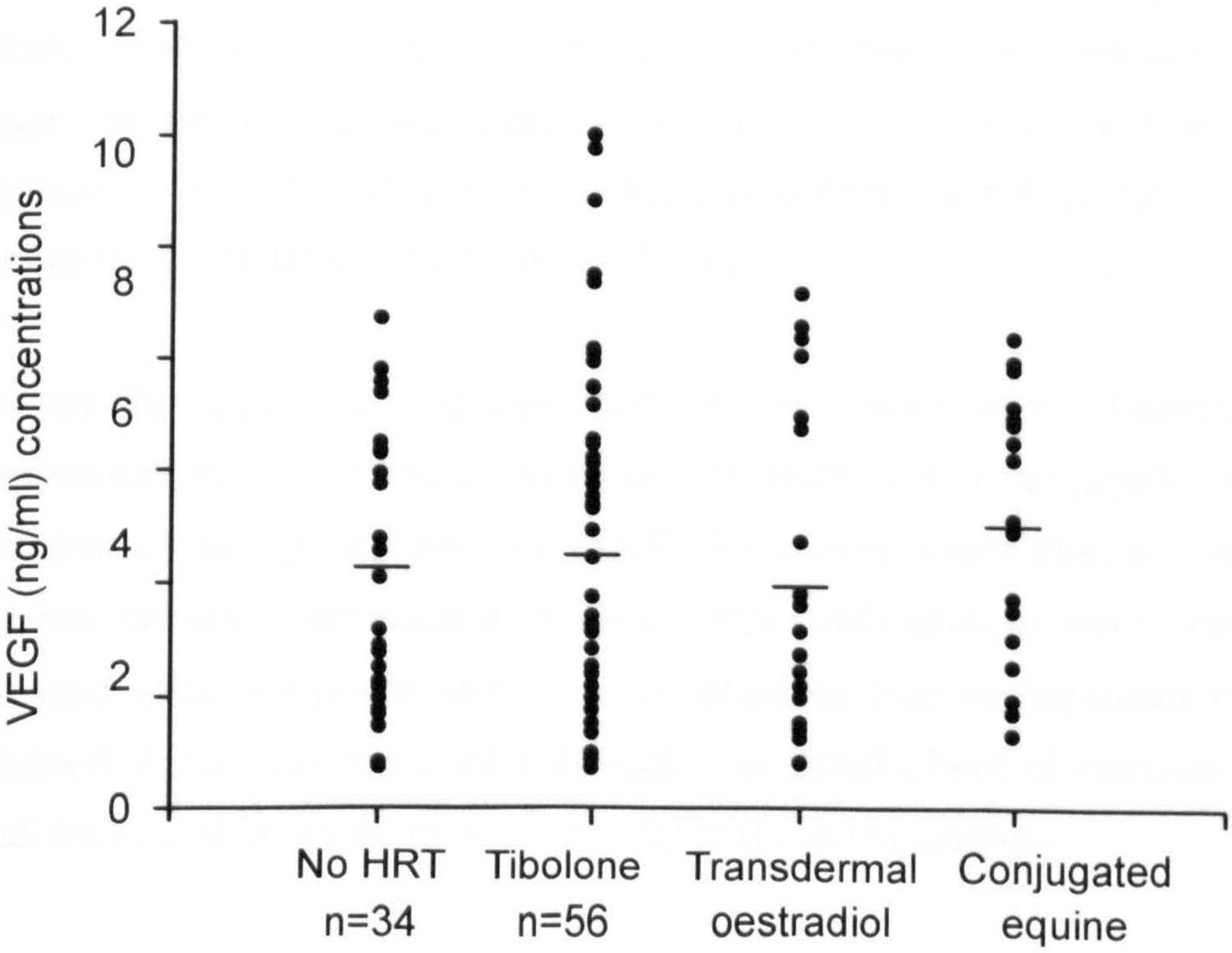


Figure 12.28

Serum VEGF concentrations in postmenopausal women with uterus in situ receiving different forms of HRT (*p=not significant).

correlated with changes in serum VEGF concentrations. Increasing blood flow velocities and decreasing arterial resistance (pulsatility and resistance indices) were observed with rising VEGF concentrations (Agrawal et al., 1999 a). Doppler blood flow studies in postmenopausal women have demonstrated increased carotid, uterine and other peripheral arterial pulsatility indices (PI), reflecting increased peripheral vascular resistance after menopause (Gangar et al., 1991, Wren, 1993, Rozenburg et al., 1994). This putative adverse effect was reversed by treatment with oestrogens (Gangar et al., 1991, Wren, 1993, Rozenburg et al., 1994). Since VEGF production in the uterus is stimulated by oestrogens (Cullinan-Bove and Koos, 1993, Hyder et al., 1996), it was hypothesised that increased blood flow velocities in the carotid, uterine and other peripheral arteries in postmenopausal women receiving HRT might be the consequence of an increase in serum VEGF concentrations.

The results of this study suggest that the uterus may contribute to serum VEGF concentrations in postmenopausal women, since mean serum VEGF concentrations were higher in untreated women with their uterus in situ compared with untreated women who had had a previous hysterectomy. It is unlikely that the ovary would have contributed to serum VEGF concentrations in postmenopausal women since there is histological evidence to suggest that ovaries from postmenopausal women do not express VEGF mRNA (Gordon et al., 1997).

The results also demonstrate that women who were treated with menopausal therapy (tibolone, transdermal oestradiol or conjugated equine oestrogens) had higher mean serum VEGF concentrations than women who were not on any menopausal therapy, when both groups were combined (with and without hysterectomy). It is possible that menopausal therapy increased VEGF concentrations through the direct effect of oestrogens on blood vessels such as peripheral, carotid and uterine arteries.

The most striking findings were the significantly higher mean serum VEGF concentrations in women who had had a previous hysterectomy who were

receiving conjugated equine oestrogens alone, when compared with other forms of therapy. This conjugated equine oestrogen mediated increase in VEGF was attenuated when women were treated with conjugated equine oestrogens with sequential norgestrel. The attenuating effect of progestogen was also observed to a similar extent, in women who were on transdermal oestradiol with sequential norethisterone acetate. It will be important to perform an experiment to substantiate this finding since the current results are based on a cross sectional study only.

Tibolone, on the other hand, which is a tissue selective synthetic steroid with mild oestrogenic, progestogenic and androgenic properties, appears to have only a modest stimulatory effect on serum VEGF. Treatment with conjugated equine oestrogens, therefore, seems to be the most potent stimulator, of the agents studied, of VEGF production. The findings of the attenuating effect of added progestogens is consistent with a recent demonstration that interrupted progestogen HRT reduced VEGF activity and subsequent angiogenesis within the human endometrium by allowing frequent progestogen withdrawal endometrial bleeding to occur (Casper, 1996). Rogers and colleagues (1993) also demonstrated that microvascular activity in the endometrium was increased by long term exposure to continuous progestogen but not by interrupted progestogen.

Although numerous reports support the role of oestrogens in cardiovascular protection (Barrett-Connor and Laakso, 1990, Stampfer et al., 1990, Barrett-Connor and Bush (1991), the magnitude of this effect and the mechanisms by which oestrogens achieve their beneficial effects on the cardiovascular function are not fully understood. The beneficial effects of oestrogens on lipid/lipoprotein profile account for, at the most, 20 - 30% of the reported cardiovascular benefits of oestrogen replacement therapy (Bush et al., 1987). Recent studies have focused to other aspects of oestrogen action which may explain the cardiovascular benefits, such as direct effects on the vessel wall, glucose metabolism and changes in fibrinolytic activity (Prelevic and Jacobs, 1997).

Coronary neoangiogenesis is an important homeostatic response to myocardial ischaemia. VEGF receptors are increased by ischaemia (Shweiki et al., 1992, Banai et al., 1994, Brogi et al., 1994, Minchenko et al., 1994, Shima et al., 1995) so allowing the effect of the increased serum VEGF to be expressed. VEGF has potent angiogenic activity and its primary role is the ability to promote endothelial growth. It has been shown to induce endothelium-dependent relaxation in both canine coronary arteries and rabbit renal arteries, both in vivo and in-vitro, most likely by stimulating nitric oxide and prostacyclin release (Ku et al., 1993, Isner et al., 1996, Morbidelli et al., 1996, Murohara et al., 1998). Intracoronary administration of recombinant VEGF in dogs also enhanced development of small coronary arteries supplying ischaemic myocardium, resulting in marked augmentation of maximal collateral blood flow delivery (Banai et al., 1994).

Angiogenic effects of exogenously administered VEGF on collateral hindlimb arterial and capillary blood flow and coronary arterial blood flow after ischaemic injury have also been demonstrated (Ferrara and Davis-Symth, 1997).

It is therefore possible that the observed oestrogen associated increase in serum VEGF concentrations, documented for the first time in the present study, could be one of the mechanisms by which oestrogens exert a beneficial effect on the cardiovascular system.

It is also possible that oestrogens mediated the effect of increasing serum VEGF concentrations in menopausal women by their direct action on peripheral, carotid and uterine arteries.

Study 6: Effect of hysterectomy and bilateral salpingo oophorectomy on serum VEGF concentrations.

Eight weeks after hysterectomy and bilateral salpingo-oophorectomy, women had lower mean VEGF concentrations (2.5 ± 0.89 ng/ml) than before hysterectomy (4.2 ± 1.2 ng/ml, $p < 0.02$) (Fig 12.29). Serum oestradiol concentrations measured 99 ± 15 pmol/L which were significantly lower than before hysterectomy and bilateral oophorectomy (138 ± 12 pmol/l, $p<0.05$). Eight weeks after hysterectomy without bilateral salpingo-oophorectomy serum VEGF concentrations were lower (3.5 ± 0.92 ng/ml) than prior to hysterectomy (4.8 ± 1.5 ng/ml, $p<0.001$) (Fig 12.29). Serum oestradiol concentrations measured 143 ± 11 pmol/l before hysterectomy and 121 ± 13 pmol/L after hysterectomy (p = not significant). Women who had bilateral oophorectomy with hysterectomy had significantly lower VEGF concentrations (2.5 ± 0.89 ng/ml) than women who had only hysterectomy without oophorectomy (3.5 ± 0.92 ng/m, $p<0.05$) (Fig 12.29). There were no differences in serum oestradiol concentrations before and after hysterectomy in women who had hysterectomy without oophorectomy.

Discussion:

The major findings of this study were that the uterus and ovary contribute to circulating serum VEGF concentrations since VEGF concentrations were significantly lower in women who had hysterectomy than before they had hysterectomy. VEGF concentrations declined further after women had hysterectomy along with bilateral oophorectomy. Serum oestradiol concentrations declined only in women having hysterectomy and bilateral salpingo oophorectomy but not in women who had hysterectomy without oophorectomy.

Although serum VEGF concentrations prior to hysterectomy were higher than VEGF concentrations in women in other published studies (Agrawal et al., 1998 a, 1998 b, 1999 a, 1999 b), perhaps this could be accounted for by the presence of menorrhagia in this group of women. Pilot studies have shown that immunoreactivity for VEGF within the uterus is increased in

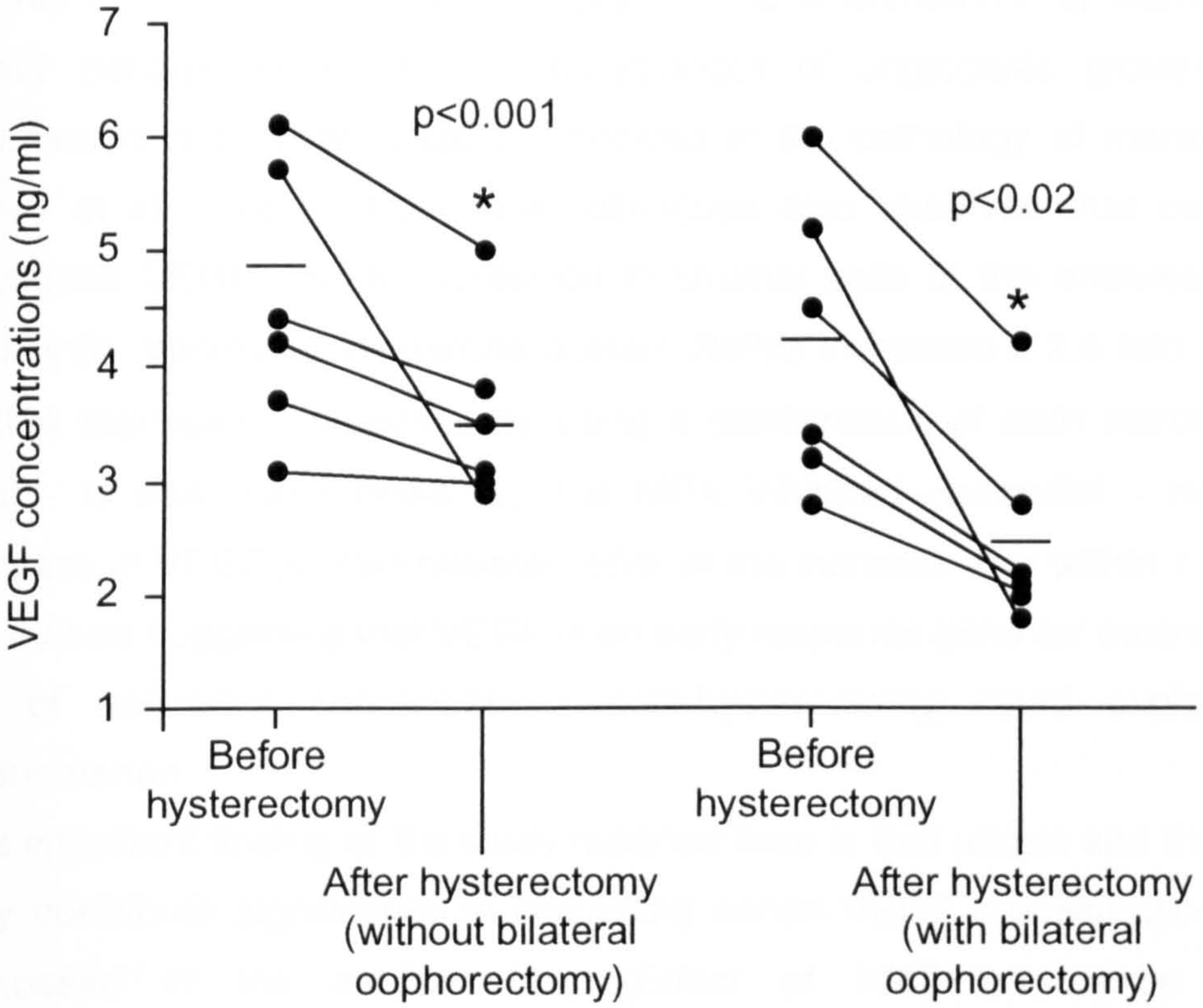


Figure 12.29

Serum VEGF concentrations in premenopausal women before and after hysterectomy (*p<0.001) and before and after hysterectomy with bilateral oophorectomy (*p<0.02). Women who had hysterectomy with bilateral oophorectomy had lower serum VEGF concentrations than women without bilateral oophorectomy (*p<0.05).

women with menorrhagia (Smith, 1998). A recent finding that proliferative activity of endothelial cells is doubled in the endometrium of women with heavy periods, suggests that disturbances of angiogenic growth factor expression or function could be involved in the pathology of menorrhagia (Kooy et al., 1996). Kooy and colleagues also observed that oestradiol increased VEGF mRNA expression in stromal cells of the endometrium 3 fold while medroxyprogesterone acetate (MPA) increased it 2.8 fold. VEGF mRNA expression increased by using a combination of both steroids (4.7 fold). It was noted however, that MPA inhibited oestradiol - mediated increase in VEGF protein release. 85% of the increase was within 1 hour of the culture suggesting that VEGF is an early response gene for oestradiol. A fall of oestradiol concentrations post-hysterectomy could explain this phenomenon.

The important finding of the study reported here is that uterus and the ovary may contribute significantly to circulating serum VEGF concentrations. As discussed in the earlier study (Effect of HRT on serum VEGF concentrations), VEGF may induce endothelium-dependent relaxation in both canine coronary arteries and rabbit renal arteries, both in vivo and in-vitro, most likely by stimulating nitric oxide and prostacyclin release (Ku et al., 1993, Isner et al., 1996, Morbidelli et al., 1996, Murohara et al., 1998). Intracoronary administration of recombinant VEGF in dogs also enhanced development of small coronary arteries supplying ischaemic myocardium, resulting in marked augmentation of maximal collateral blood flow delivery (Banai et al., 1994).

It is therefore possible that the fall of serum VEGF (and correspondingly of circulating serum oestradiol after oophorectomy) documented for the first time in the present study, could be one of the reasons why the incidence of cardiovascular disease increases after menopause or after hysterectomy and oophorectomy. The findings therefore, also support the hypothesis that VEGF and oestrogen contribute to a cardio-protective effect in premenopausal women, who have a lower incidence of coronary artery disease. The study reported here supports the evidence that the uterus and ovaries contribute to serum VEGF in healthy women.

Study 7: VEGF release by cultured granulosa lutein cells.

In this study VEGF released by granulosa lutein cells cultured in-vitro was observed. The effect of incubation of granulosa cells with various hormones on VEGF release and differences in the VEGF release by granulosa cells obtained from women with PCO and those who developed OHSS compared with those obtained from women who had normal ovaries and those who did not develop OHSS, was observed.

Effect of different hormones on the release of VEGF:

These observations are based on analysis of cell culture samples obtained from 4 women, two with normal ovaries and two with PCO. Incubation with FSH, LH and hCG enhanced VEGF release by luteinised granulosa cells at all concentrations studied (i.e., 0.1, 0.5, 1 and 5 iu/ml). Although incubation with insulin alone did not augment VEGF release, addition of insulin augmented hCG stimulated release of VEGF. This effect of insulin augmentation on hCG stimulated cells was also observed in all 20 women. There were no differences in VEGF release achieved by stimulation with FSH, LH and hCG (Fig 12.30).

No significant release of VEGF in the culture medium in control cells (Control) was observed. Addition of testosterone to the culture did not affect VEGF release (i.e., Control + Testosterone) (Fig 12.30).

PCO versus normal ovaries:

These observations are based on analysis of cell culture samples obtained from 20 women, 10 with normal ovaries and 10 with PCO/PCOS. Under all culture conditions studied, VEGF released from granulosa lutein cells obtained from women with PCO/PCOS was significantly greater ($p<0.01$) than observed from cells obtained from women with normal ovaries (Fig 12.31).

OHSS versus no OHSS development:

Women in this study with normal ovaries did not develop OHSS. Amongst

women who had PCO/PCOS (n = 10), VEGF released by granulosa lutein cells obtained from women who subsequently developed OHSS (n = 4) was higher than that observed from women who did not develop OHSS (n = 6) (Fig 12.32).

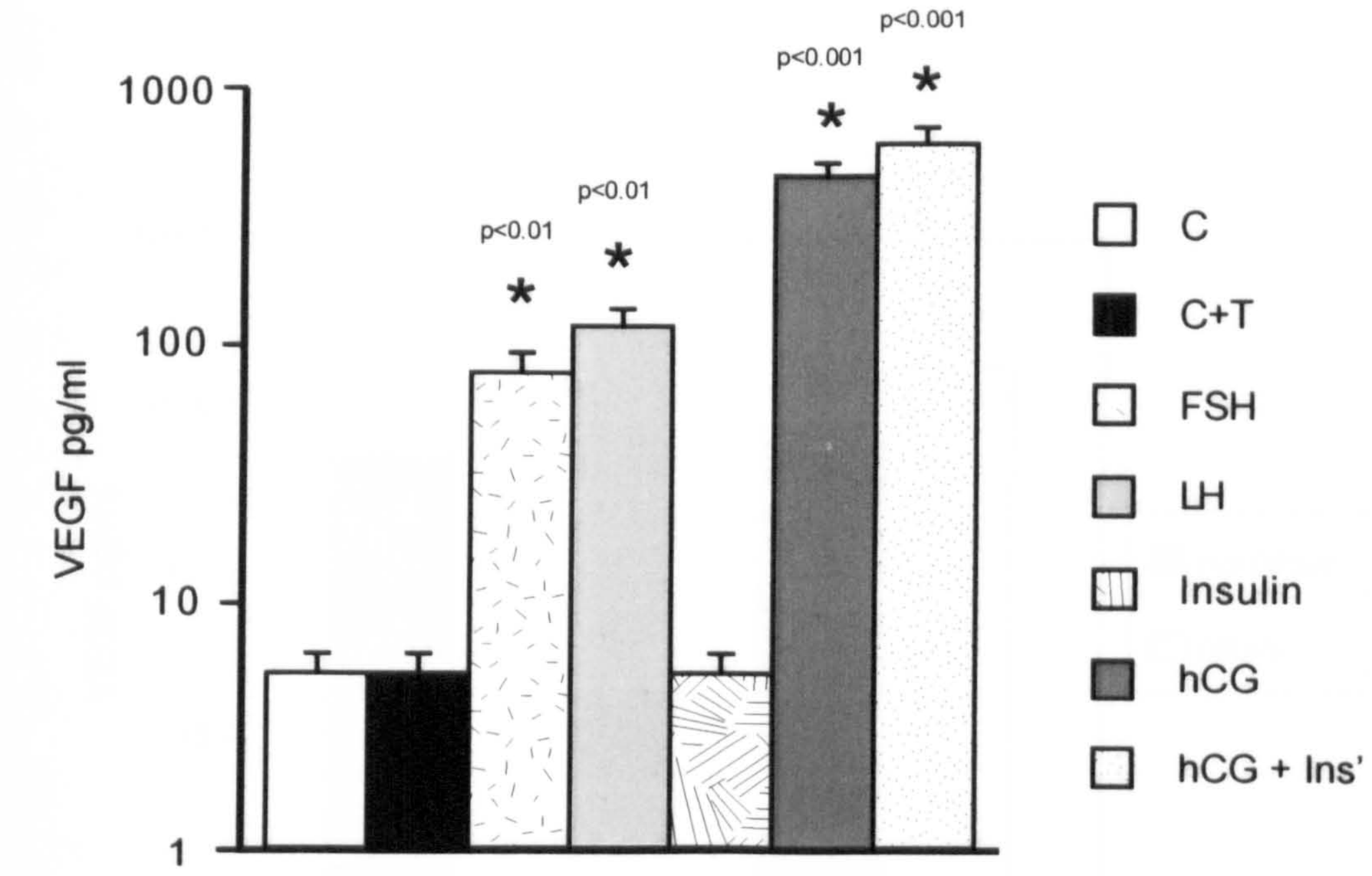


Figure 12.30

VEGF release by granulosa cells after 4 days of incubation with control (no hormones), C+T (cells with addition of testosterone), cells with addition of FSH, LH, insulin, hCG and hCG + Insulin (Ins') at a concentration of 1 iu (n=4).

Figure 12.31
VEGF release by incubation of granulosa cells obtained from women with normal ovaries (n=10) and PCO (n=10) without hormones and with hormones (c = control, C+T = control + testosterone), FSH, LH, hCG and hCG + Insulin (Ins') at a concentration of 1iu (n=20, $p<0.01$).

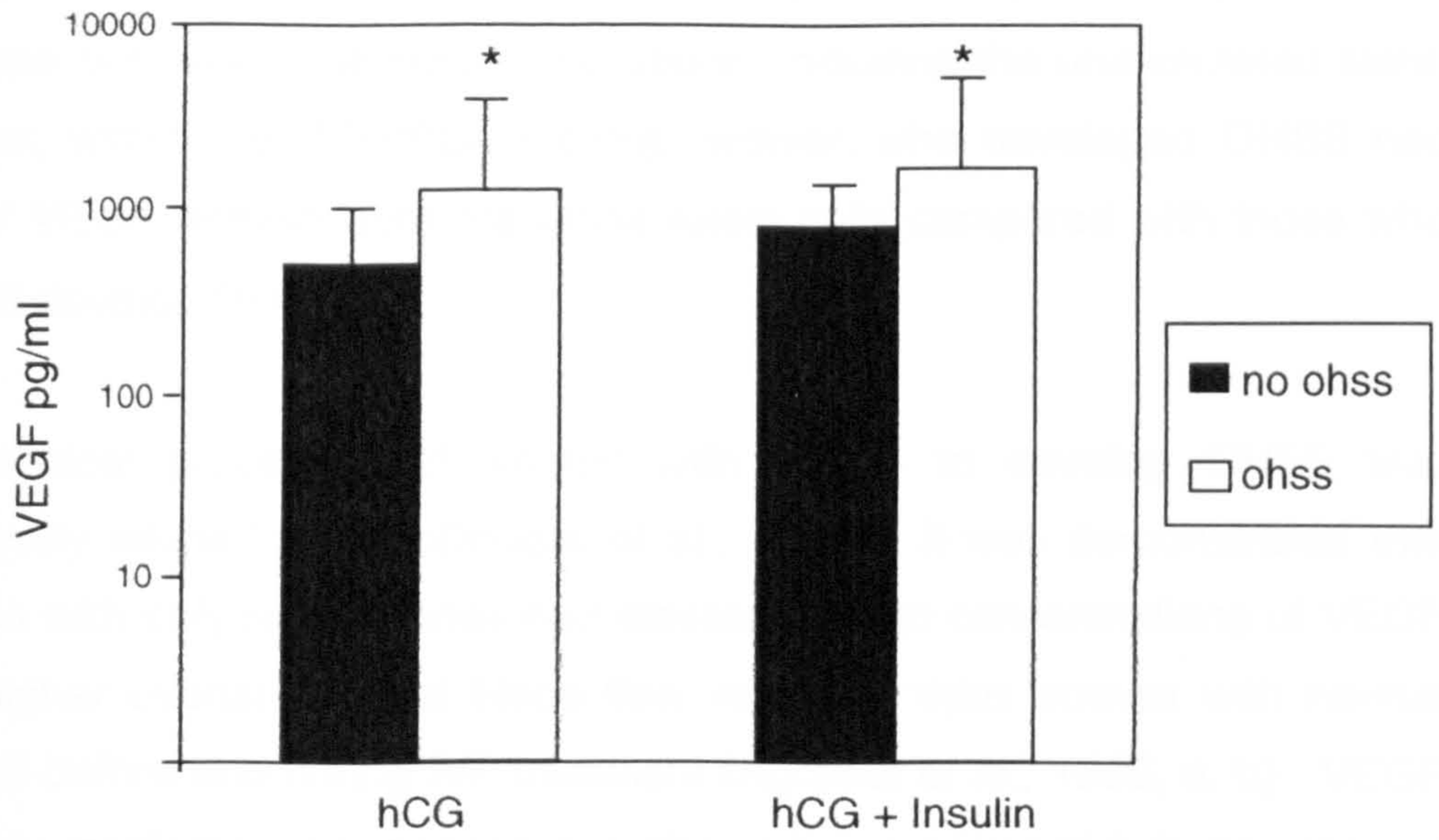


Figure 12.32

VEGF release by granulosa lutein cells obtained from women with PCO (n=10) who developed OHSS (n=4) and those who did not develop OHSS (n=6) after 4 days of incubation with hCG and hCG + Insulin at a concentration of 1 iu (*$p<0.001$).

Discussion:

This study demonstrated that the amount of VEGF released was greater from cultured granulosa lutein cells obtained from women with PCO/PCOS compared with those obtained from women with normal ovaries. This increase occurred in all culture conditions, including the unstimulated state. Further, within the PCO/PCOS group, women who developed OHSS had higher VEGF release from granulosa lutein cells compared with those who did not develop OHSS.

The clinical propensity of women with PCOS to develop OHSS was previously alluded to (MacDougall et al., 1993). It was demonstrated that women with polycystic ovaries had elevated serum concentrations of VEGF and higher ovarian stromal blood flow velocities than women with normal ovaries before and during IVF treatment (Agrawal et al., 1998, a, b). VEGF not only mediates angiogenesis but also induces connective tissue stromal growth by increasing microvascular permeability. The extravascular matrix thus formed favours the ingrowth of new blood vessels, which in turn organises the avascular fibrin matrix into a highly vascularised connective tissue stroma (Connolly et al., 1991, Kamat et al., 1995). These observations and the findings of elevated serum VEGF concentrations in women with PCO/PCOS resonate with the immunohistochemical demonstration, of extensive VEGF staining in the hyperthecotic stroma of the PCO (Kamat et al., 1995). Since serum VEGF concentrations correlate with ovarian stromal blood flow, these changes in blood may have significance in relation to the characteristically exuberant response to stimulation of the PCO/PCOS. Second, the raised levels of VEGF occurred in women with PCO as well as those with PCOS, suggesting that it is a constitutive feature of PCO rather than a result of stimulation by luteinising hormone.

The expression of VEGF mRNA in granulosa lutein cells was demonstrated by Yan et al., 1993 and Doldi et al., 1997. A positive correlation was observed between serum oestradiol and progesterone concentrations on the day of oocyte retrieval and VEGF mRNA expression in granulosa lutein cells (Doldi et al., 1997). They also observed that the VEGF mRNA expression

was significantly higher in granulosa cells obtained from women with higher number of oocytes and higher fertilisation rates than obtained from women with lower number of oocytes and a lower fertilisation rate. It is known that women with PCO recruit greater number oocytes compared with women with normal ovaries (MacDougall et al., 1993). The study of Doldi et al. 1997, was consistent with the observation that VEGF mRNA expression was higher in women with PCO compared with normal ovaries.

In the present study it was also demonstrated that VEGF was released by cells incubated with FSH, LH, hCG and by addition of insulin to hCG stimulated cells. The VEGF release following stimulation with LH or hCG are consistent with the results previously observed (Sweiki et al., 1993, Koos et al., 1995, Neulen et al., 1995, Christenson and Stouffer, 1997) and the findings of the clinical studies 1, 2, 3 and 4. Under the conditions of culture reported here VEGF concentrations after stimulation with FSH and LH were similar to those observed after stimulation with hCG.

Unstimulated cells and cells stimulated with insulin alone, did not produce significant release of VEGF. Addition of testosterone to cells unstimulated with other hormones did not alter VEGF concentrations. This finding suggests that testosterone may not mediate VEGF release from granulosa cells.

In the present study, significant VEGF release was detected 48 hours after culture and rose for 4 days. VEGF release then declined steadily until 12 days of culture after which VEGF release became undetectable.

Studies of Lee et al., 1998 and Antczak et al., 1997, demonstrated similar findings to those reported here. Lee et al., 1998, demonstrated that VEGF concentrations in cultures rose from day 1 to a maximum on day 3 and then declined. However addition of hCG increased the production of VEGF 3 fold after 4 days of incubation with hCG. They also found that co-incubation with trilostane, which inhibits of 3-beta steroid dehydrogenase reduced progesterone production by the cultured cells but had no effect on VEGF

production. This observation suggests that hCG stimulated production of VEGF is independent of progesterone synthesis.

hCG has also been shown to augment VEGF mRNA expression in cultured human granulosa lutein cells in a time and dose dependent manner. Neulen et al., 1998 observed that in cells without the addition of hCG, VEGF expression steadily declined after 4 days of the ovulatory stimulus and by day 10 it was reduced to 30% of the value at day 4. With an additional stimulus of 1 iu / ml hCG, however, the expression of VEGF was 4 times greater than control and increased 100% from day 4 to day 10. Their results suggested that VEGF production by granulosa cells may not be regulated by product inhibition. Higher concentrations of hCG therefore provoked a greater production of VEGF. They considered that for this reason, women who became pregnant and the ones with multiple pregnancies were at a higher risk of OHSS compared with women who did not achieve pregnancy or who had singleton pregnancies (Mathur et al., 1995).

It was also observed that insulin augmented release of VEGF in response to incubation with hCG. The mechanism is not known. However this small significant difference in VEGF release with addition of insulin to hCG stimulated cells may not be physiologically significant.

It has previously been established that preincubation with insulin significantly increased the subsequent responsiveness of human granulosa cells to LH and to FSH (Willis D. 1997, Adashi et al., 1985). It was speculated that insulin mediated this action by increasing LH receptor number, the affinity of LH to its receptor or post receptor events. However investigations in the porcine and murine ovaries have demonstrated that insulin and IGF-1 increase LH receptor number and not the affinity of LH for the receptor (May et al., 1980, Adashi et al., 1985). It is possible that addition of insulin to hCG stimulated cells augmented VEGF release by a similar mechanism. In the present study however, fetal calf serum was used in the complete medium for granulosa cell culture to facilitate the cells to stick to the plates during incubation. The role of insulin in this study is therefore, not defined.

Studies by Willis et al., 1997 demonstrated no consistent effect of insulin on granulosa lutein cell steroidogenesis. They speculated that the variable effect might reflect the degree of luteinisation of the granulosa cells from different patients. Based on the results of their studies and those reported elsewhere (Hurwitz et al., 1987, Bergh et al., 1991, Hill and Osteen 1992, Poretsky et al., 1996) they concluded that granulosa cells obtained from women undergoing ovarian stimulation were unsuitable for comparing insulin effects on human granulosa lutein cells from women with normal and polycystic ovaries. It has therefore been concluded that luteinised granulosa cells were an appropriate model for studying corpus luteal function rather than granulosa cells' activity.

I conclude that VEGF released by granulosa lutein cells is gonadotrophin dependent and augmented by insulin. The raised circulating concentrations of VEGF in women with PCOS may not only be due to an increased number of actively secreting granulosa lutein cells but also due to increased secretory capacity of each granulosa cell.

Summary & Conclusions

SUMMARY & CONCLUSIONS:

The first study established a link between serum VEGF and cyclical changes within the uterus and the dominant ovary. The link was demonstrated by the correlation of serum VEGF concentrations with changing Doppler blood flow velocities during the normal menstrual cycle and the positive correlation with serum progesterone and oestradiol concentrations in the midluteal phase. No differences in serum VEGF concentrations were observed between healthy men and premenopausal women in the early follicular phase of the menstrual cycle.

The second study demonstrated that the increased ovarian stromal blood flow demonstrated by Doppler blood flow velocity measurements in women with polycystic ovaries was associated with higher serum concentrations of VEGF. Since VEGF increases vascular permeability, this growth factor may contribute to the formation of the increased s

women who did not. The results may also help to explain the higher risk of OHSS in women with PCO and PCOS compared with women normal ovaries.

Serum VEGF concentrations were higher 2 weeks after embryo transfer in women who achieved pregnancy compared with women who did not achieve pregnancy.

In the fourth study, the results indicated that VEGF may provide an important nonsteroidal index of the ovarian response to gonadotrophin stimulation. This result is biologically plausible since VEGF is probably a mediator of OHSS and independent of the other markers of OHSS. Measurements of serum VEGF concentrations on the day of hCG administration and on the day of egg collection, along with a count of total follicles on the day of hCG administration and a pre-treatment diagnosis of PCO, may have an advantage over serum oestradiol concentrations. Use of serum E_2 measurements as an index of ovarian response is becoming less reliable as schedules of ovarian stimulation move from combined gonadotrophin preparations to FSH containing preparations alone, because serum E_2 concentrations may underrepresent the degree of follicular response to ovarian stimulation. Further prospective studies will be required to define the most reliable cutoff - points for the prediction and therefore ultimately the prevention, of OHSS.

In the fifth study, it was reported that mean serum VEGF concentrations were higher in postmenopausal women with their uterus in situ compared with women who had had a previous hysterectomy and were not receiving any HRT. In addition, mean serum VEGF concentrations were higher in postmenopausal women receiving HRT, particularly those on conjugated equine oestrogens alone. It is possible that this increase in VEGF concentrations contributes to the beneficial effects of menopausal therapy on the cardiovascular system. It seems however, that the addition of sequential progestogen attenuates the increase in serum VEGF concentration observed with treatment with conjugated equine oestrogens

alone.

In the sixth study, it was reported that serum VEGF concentrations fell rapidly after hysterectomy and further, after hysterectomy along with bilateral salpingo - oophorectomy in healthy pre -menopausal women, supporting the possibility of the uterus and ovaries contributing to serum VEGF in healthy women.

In the seventh study, it was concluded that VEGF released by granulosa lutein cells is gonadotrophin dependent and is augmented by insulin. VEGF released from granulosa cells stimulated or unstimulated with hormones, obtained from women with PCO and those who developed OHSS was greater than those obtained from women with normal ovaries and those who did not develop OHSS. The raised circulating concentrations of VEGF in women with PCOS may not only be due to increased number of actively secreting granulosa lutein cells but also due to increased secretory capacity of each granulosa cells.

Conclusions:

In this thesis it was demonstrated that the female reproductive tract is a major source of circulating VEGF in women by studying variations during the menstrual cycle (Agrawal et al., 1999 a) and before and after hysterectomy (Agrawal et al., 2000). The clinical propensity of women with PCOS to develop OHSS was also alluded to. It was therefore not surprising that in the study of VEGF concentrations in women undergoing IVF treatment, women with polycystic ovaries had elevated serum concentrations of VEGF and higher ovarian stromal blood flow velocities than women with normal ovaries before and after treatment commenced and during the phase of ovarian stimulation (Agrawal et al., 1998 a, b).

VEGF not only mediates angiogenesis but also induces connective tissue stromal growth by increasing microvascular permeability, which leads to extravasation of plasma proteins (Connolly, 1991). The extravascular matrix

thus formed favours ingrowth of new blood vessels and fibroblasts, which in turn organises the avascular provisional fibrin matrix into a mature, vascularised connective tissue stroma. These observations and our findings of elevated serum VEGF concentrations in women with PCO resonate with the immunohistochemical demonstration by Kamat et al., 1995, of extensive VEGF staining in the hyperthecotic stroma of the PCO.

There is also evidence that angiogenic factors like bFGF, TGF beta, PDGF and nitric oxide act as agonists to the action of VEGF within the ovary (Connolly 1991). Unlike other growth factors responsible for angiogenesis, e.g. bFGF, which is largely intracellular and nondiffusible, VEGF, is a soluble diffusible growth factor. VEGF therefore is a more likely mediator of angiogenesis.

The significance of these observations seems considerable. First, it is of great interest to note that serum VEGF concentrations correlate with ovarian stromal blood flow, as measured by colour Doppler ultrasound (Agrawal et al., 1998 a). These changes in blood flow demonstrated by Doppler ultrasound were found to be raised in the ovarian stroma of women with PCO by Zaidi et al., 1995, and later confirmed in study two, may have significance in relation to the characteristically exuberant response to stimulation of the PCO. Second, the raised levels of VEGF occurred in women with ultrasound detected PCO as well as those with PCOS, suggesting that it is a constitutive feature of PCO rather than a result of stimulation by luteinising hormone (LH).

It was also considered whether increased ovarian circulating androgens in women with PCO could be a cause of the increased vascularity. In support of this hypothesis there are recent data indicating testosterone increased VEGF expression within the prostate gland and castration decreased testicular and prostatic VEGF (Frank-Lissbrant et al., 1998). Positive correlation between serum VEGF concentrations and testosterone concentrations were observed in the clinical studies (Agrawal et al., 1999 a). It was therefore hypothesised that ovarian hyperandogenism in women with

PCO increased VEGF expression within the ovary.

There is a striking difference in the ovarian response to gonadotrophic stimulation of women with amenorrhoea and hypogonadotrophic hypogonadism and normal ovaries compared with women with PCO. This is most clearly demonstrated in a report by Shoham et al., 1992, which exemplifies the notion that the essential feature of response of the PCO to gonadotrophic stimulation is loss of normal intraovarian autoregulatory processes that underlies the emergence of a single dominant follicle with suppression of cohort follicles. It is this persistence of cohort follicles, despite the development of a dominant follicle in response to treatment that underlies the risk of OHSS and multiple pregnancy, to which women with PCO are so prone.

The question therefore arises as to what are the important factors that underlie the intraovarian autoregulation that results in unifollicular ovulation. Clearly there are endocrine factors to consider such as the amount and the potency of gonadotrophins to which the ovary is exposed during ovulation induction. Intraovarian paracrine factors are also considered important, particularly the concentration of insulin - like growth factor - 1, which is known to augment the granulosa cell response to FSH (Adashi et al, 1985). During the development of a dominant follicle, blood is normally directed to the ovary bearing the dominant follicle and within the ovary there is diversion of blood flow towards the dominant follicle (Sladkevicius et al., 1993). Presumably this change in intraovarian blood flow contributes to concentration of FSH in the dominant follicle. Correspondingly the relative lack of FSH in the cohort follicles presumably contributes to their atresia. The excessive amount of VEGF within the stroma of PCO may result in a failure of diversion of blood flow away from the cohort follicles to the leading follicle, thereby permitting uninhibited growth of other follicles and a multifollicular response (Agrawal et al., 1998 a). These findings provide a mechanism that helps to explain the link between VEGF, OHSS and PCO.

The two possible explanations for hypersecretion of VEGF in women with

PCOS could be either that there is an increase in the number of VEGF secreting cells or secondly that the cells may individually hypersecrete VEGF.

In the laboratory study of this thesis it was demonstrated that VEGF release by granulosa lutein cells into cell culture media after the cells were incubated with FSH, LH, hCG and insulin were higher from granulosa cells obtained from women with PCO compared with those obtained from women with normal ovaries under comparable culture conditions.

The findings outlined above, place VEGF at the centre stage, as a constitutive feature of PCO, as the underlying cause of loss of intraovarian regulation and as a mediator of the major complications of ovarian stimulation. Its intimate involvement in the process of neovascularisation makes it an attractive candidate for the pivotal place in the pathophysiology of PCO / PCOS and OHSS.

Its role in the angiogenic process of the endometrium may provide a useful clinical tool in increasing angiogenesis within the endometrium in women undergoing ovarian stimulation cycles for conception.

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ORIGINAL PAPERS

CLINICAL EXPERIENCE

Severe ovarian hyperstimulation syndrome: serum and ascitic fluid concentrations of vascular endothelial growth factor

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Abbreviations

ELISA	enzyme-linked immunosorbent assay
GnRH	gonadotrophin-releasing hormone
hCG	human chronic gonadotrophin
IVF	in-vitro fertilization
LH	luteinizing hormone
OHSS	ovarian hyperstimulation syndrome
VEGF	vascular endothelial growth factor

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Introduction

We describe a case of severe ovarian hyperstimulation syndrome (OHSS) which occurred during in-vitro fertilization (IVF) treatment. The role of vascular endothelial growth factor (VEGF), a recently recognized mediator of OHSS, is also described.

Case report

A 29-year-old patient with secondary infertility of 6 years' duration underwent IVF treatment. She had had a regular menstrual cycle until the age of 23 years when she conceived spontaneously. Unfortunately the pregnancy miscarried. Subsequently her periods became infrequent (every 10 to 12 weeks), and since then, she had been unable to conceive.

Physical examination was unremarkable and her body mass index was 22.2 kg/m^2. Laboratory investigations showed normal serum concentrations of gonadotrophins, testosterone, prolactin and thyroid hormones. Her fallopian tubes were confirmed to have normal patency at diagnostic laparoscopy and an ultrasound examination of her pelvis revealed polycystic ovaries. She was treated with 12 cycles of ovulation induction with clomiphene citrate without success. She was then referred for IVF treatment, which commenced in May 1996. Pituitary desensitization was achieved with the standard 'long' protocol of treatment [1] with Dtrp6 Pro9 methylamide gonadotrophin-releasing hormone (GnRH) analogue (Regulatory Peptide Unit, Cape Town, South Africa). Ovarian stimulation was achieved using Humegon (distributed by Donmed Pharmaceuticals Ltd, Bedfordview, South Africa), which contains 75 IU of luteinizing hormone (LH) and of follicular-stimulating hormone activity per ampoule. A dose of 300 IU was administered for 3 days, followed by 150 IU for a further 6 days and 75 IU for 1 day. Follicle growth was monitored using vaginal ultrasound.

The patient showed a multifollicular response, and the serum oestradiol level on the day of administration of human chorionic gonadotrophin (hCG) (day 11) was 45 229 pmol/l. hCG (Serono Research Labs SA, Parkwood, South Africa) was administered at a dose of 5000 IU. Transvaginal oocyte retrieval was performed 34 h after administering hCG, under ultrasound guidance, using general anaesthesia. Of the 26 oocytes retrieved, 20 were fertilized. Since the patient had a very high serum concentration of oestradiol and was at a risk of developing ovarian hyperstimulation, embryo transfer was deferred and the patient asked to continue treatment with the GnRH analogue.

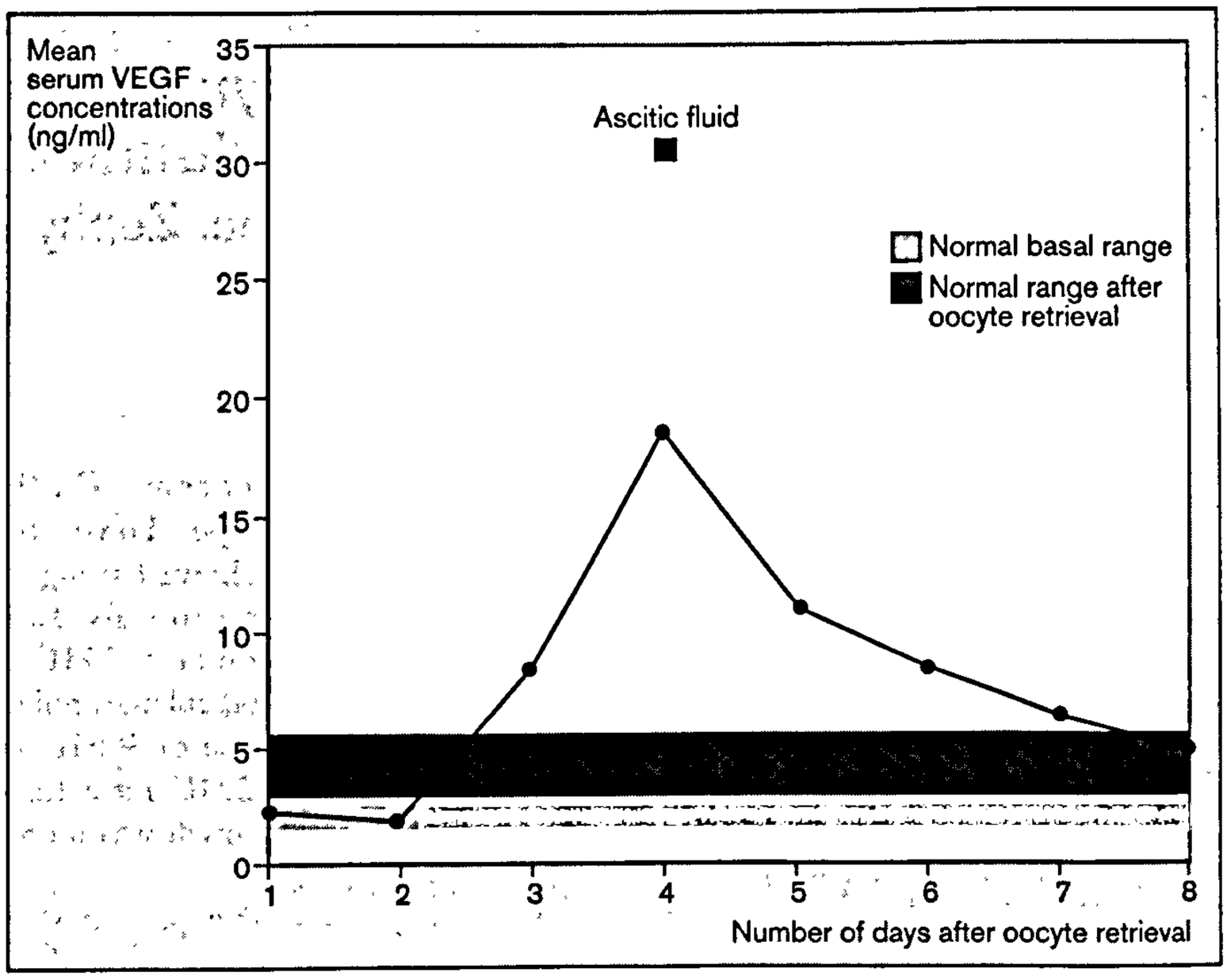
She was admitted into hospital on the day of egg collection for observations. Serum urea and electrolytes, liver function tests, haematocrit, clotting profile, renal profile and progesterone concentrations were monitored for the subsequent 16 days. These were within normal limits. Serum VEGF concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) (Peninsula Labs Inc, USA) following oocyte retrieval on the days indicated in Figure 1.

The patient was asymptomatic until the third day after egg collection, but an ultrasound examination on that day showed free fluid in her abdomen. On the following day she developed abdominal discomfort, distention, nausea, vomiting and respiratory difficulties. On examination, she was found to be severely ketotic and had massive ascites. Examination of her chest was normal.

Paracentesis was performed over the next 2 days. Ascitic fluid VEGF concentration, as measured by ELISA, was

Figure 1

Rise of serum vascular endothelial growth factor (VEGF) after oocyte retreival.



raised at 31.2 ng/ml (Fig. 1). Treatment also included maintenance of adequate intravascular volume and transfusion of fluids and fresh frozen plasma to increase intravascular oncotic pressure. Her condition rapidly improved and she was discharged 16 days after the oocyte retrieval procedure.

Discussion

Ovarian hyperstimulation syndrome (OHSS) is one of the most serious iatrogenic complications of ovulation induction. Its pathophysiology is unknown, treatment is non-specific and the mainstay of management is its prevention [2].

Our patient developed severe OHSS (Table 1), which is characterized by marked cystic ovarian enlargement and massive extravascular fluid shifts, giving rise to protein-rich ascites, pleural effusion and pericardial effusion. Loss of protein causes a fall in the plasma oncotic pressure and secondary intravascular fluid volume depletion. The haemoconcentration poses a risk of thromboembolism. There is suppression of urine formation and renal failure may ensue [3].

The known risk factors for the development of the syndrome [4] are:

(1) presence of polycystic ovaries (not necessarily polycystic ovary syndrome);
(2) multiple small and intermediate-sized follicles;
(3) young slim patient;
(4) exposure of the ovaries to hCG or LH (especially a higher dose of hCG);
(5) use of GnRH analogues during ovulation induction;
(6) high dose and duration of treatment with gonadotrophins;
(7) pregnancy.

As described in the case history, this patient had polycystic ovaries. Such ovaries are known to show a multifollicular rather than a unifollicular response to ovarian stimulation, with the development of multiple small and intermediate-sized follicles, an important predisposing factor for the development of the syndrome [5]. Polycystic ovaries are present in 20% of normal women but they occur in 40% of patients undergoing IVF, irrespective of the indication for treatment [5].

Table 1. Classification of ovarian hyperstimulation syndrome

Degree	Signs and symptoms
Mild	Weight gain, thirst, nausea, abdominal discomfort, mild abdominal distention, ovaries larger than 5 cm in diameter
Moderate	Nausea, vomiting, abdominal discomfort and pain, dyspnoea, abdominal distention but not tense ascites detected by ultrasound (but not clinically evident)
Severe	Evidence of intravenous fluid loss, third-space fluid collection, e.g. ascites, pleural or pericardial effusion, haemoconcentration, hypovolaemia, oliguria, hepato-renal failure.

The patient was given hCG at a dose of 5000 IU. It is exposure of the ovaries to LH or hCG that mediates the neovascularization and increased vascular permeability [6] that underlie transformation of the avascular Graffian follicle into a corpus luteum. If the ovaries are not exposed to LH or hCG, OHSS does not occur. In women having ovarian stimulation using a GnRH analogue, OHSS is almost always preventable by withholding hCG. OHSS is therefore always iatrogenic.

Pituitary desensitization with superactive GnRH agonists protects the ovary from an endogenous LH surge, thereby providing the flexibility of scheduling ovum pick-up on convenient days. The protection so afforded renders the ovary more amenable to stimulation of multifollicular development by high-dose gonadotrophin treatment. Not surprisingly this very advantage makes ovarian hyperstimulation more common in programmes utilizing pituitary desensitization [7].

Although it has been known for many years that high circulating concentrations of oestradiol are a marker of the syndrome, oestradiol itself is not the cause of sudden increase in the vascular permeability of OHSS. Such a change is not, after all, a feature of treatment with oestrogen itself, even when the levels rise very abruptly as after an implant. As a predictor of OHSS, oestradiol predicts OHSS in only a quarter of the cases [8]. OHSS can in fact occur in the virtual absence of oestradiol secretion [9].

VEGF is a recently recognized mediator of increased vascular permeability associated with OHSS [10]. The other two factors possibly mediating OHSS are the ovarian renin–angiotensin system [11] and the ovarian cytokines [12]. VEGF is an endothelial-cell mitogen and multifunctional cytokine with potent angiogenic properties. It is a heparin-binding, dimeric, disulphide-bonded glycoprotein with a molecular mass of approximately 46 kDa. It is structurally related to platelet-derived growth factor. Besides stimulating proliferation and migration of endothelial cells, VEGF also renders these cells hyperpermeable so that they spill plasma proteins into the extravascular space. It is therefore also called vascular permeability factor [13].

The known human receptors for VEGF are cell-surface, membrane-spanning, tyrosine kinase receptors which are expressed predominantly on endothelial cells. VEGF is produced and secreted by several normal and abnormal cell types, including the hyperthecotic stroma of polycystic ovaries, in which it may play a pathophysiological role. In the granulosa cell compartment, high levels of VEGF messenger RNA are only detectable at the immediate preovulatory stage. Shortly after ovulation, or after exposure of the dominant follicle to hCG, granulosa cells are transformed into lutein cells and the development of the corpus luteum ensues. The predominant site of VEGF expression is then the lutein cells. The increase in VEGF messenger RNA is apparent 1–4 h after hCG treatment and the cells continue to express VEGF in the fully developed corpus luteum. Women who recruit an excess number of follicles overexpress VEGF, which may be responsible for the fluid shift from the vascular bed to the extravascular space which characterizes this syndrome of OHSS [14].

High levels of VEGF, as measured by bioassay, have been found in the ascitic fluid of women who developed OHSS compared with the ascitic fluid of women who did not develop the syndrome [10]. In addition, high serum VEGF levels have also been found in patients who developed the syndrome [15].

The development of an ELISA assay has offered the possibility of exploring VEGF levels in patients undergoing ovarian stimulation and relating the levels to the risk of OHSS. As shown in Figure 1, we found high levels of VEGF in the serum and ascitic fluid of our patient. In addition, rising serum VEGF levels simultaneously with the increasing severity of OHSS were clearly demonstrated. We have also found high levels of VEGF in the pleural and ovarian follicular fluid of women who developed OHSS (unpublished data).

Conclusion

The patient developed severe OHSS. The condition is a cause of substantial morbidity and occasional mortality. VEGF emerges as an important mediator of this syndrome but whether it will provide an index of the risk of development of the syndrome is yet to be explored.

References

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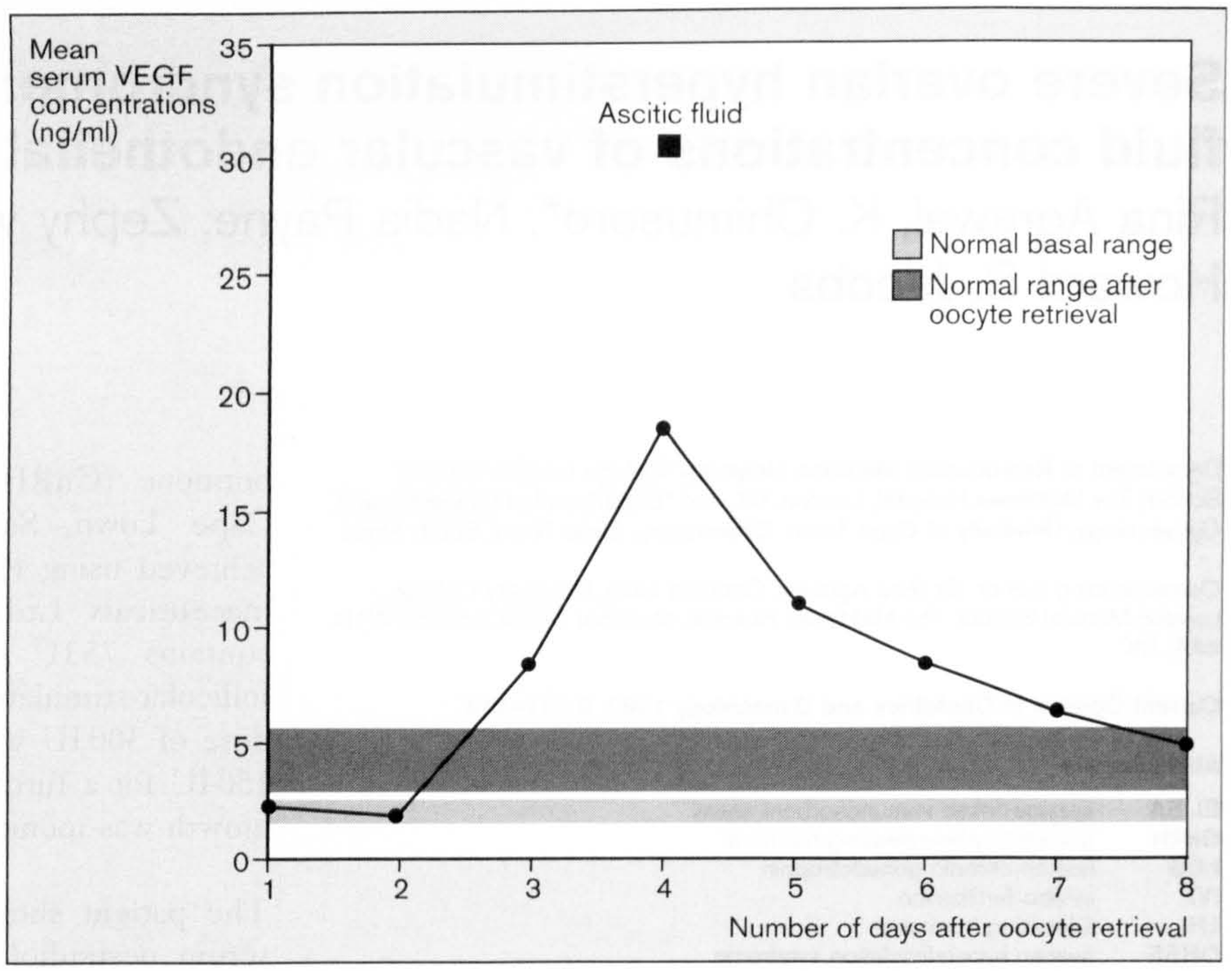

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Serum vascular endothelial growth factor concentrations and ovarian stromal blood flow are increased in women with polycystic ovaries

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The aim of this study was to determine basal serum vascular endothelial growth factor (VEGF) concentrations and Doppler blood flow changes within the ovarian stroma of women with polycystic ovaries (PCO) and women with normal ovaries. Pulsed and colour Doppler blood flows within the ovarian stroma were recorded, and serum VEGF concentrations measured, in the early follicular phase (days 2–3 of a menstrual cycle) in 60 women undergoing ovarian stimulation for in-vitro fertilization. 36 women had normal ovaries, 14 women had PCO as seen on pelvic ultrasound examination and 10 had polycystic ovarian syndrome (PCOS). Mean ± SD serum VEGF concentrations were significantly higher ($P < 0.001$) in women with PCO and PCOS (3.4 ± 0.7 and 3.2 ± 0.66 ng/ml respectively) compared with women with normal ovaries (2.3 ± 0.5 ng/ml). Mean peak systolic blood flow velocity (PSV) and time-averaged maximum flow velocity (TAMXV) were significantly higher ($P < 0.001$) in women with PCO and PCOS compared with women with normal ovaries. The mean PSV were 15 ± 4 and 16 ± 4 cm/s in women with PCO and PCOS respectively, compared with 9 ± 2 cm/s in women with normal ovaries. The TAMXV were 9 ± 3 and 11 ± 3 cm/s in women with PCO and PCOS respectively compared with women with normal ovaries (5.8 ± 1.5 cm/s). Serum VEGF concentrations were positively correlated with PSV ($r = 0.44$, $P = 0.001$) and TAMXV ($r = 0.45$, $P < 0.000$) in all three groups of women. Higher serum concentrations of VEGF in women with PCO and PCOS may relate to the increased vascularity that underlies the increased blood flow demonstrated by Doppler blood flow velocity measurements in these women. The results may explain the higher risk of ovarian hyperstimulation syndrome in programmes of ovarian stimulation in patients with PCO compared with those with normal ovaries.

Key words: Doppler blood flow velocity/polycystic ovarian syndrome/polycystic ovary/vascular endothelial growth factor

Introduction

Several growth factors have been implicated in angiogenesis within the ovary, including basic fibroblast growth factor (bFGF; Gospodarowicz, 1974), transforming growth factor β (TGF-β; Pepper *et al.*, 1993) and platelet-derived growth factor (PDGF; Klagsbrun and D'Armore, 1991; Folkman and Shing, 1992). Luteinizing hormone (LH)-induced neoangiogenesis has also been described (Findlay, 1986).

Vascular endothelial growth factor (VEGF), an endothelial cell mitogen with potent angiogenic properties, is emerging as an important mediator of neoangiogenesis (Senger *et al.*, 1983; Ferrara and Henzel, 1989; Gospodarowicz *et al.*, 1989; Leung *et al.*, 1989; Phillips *et al.*, 1990; Connolly, 1991). VEGF mRNA has a wide distribution in normal and malignant tissue (Senger *et al.*, 1993) and is highly expressed in areas of active vascular proliferation. Unlike other growth factors, the mitogenic activity of VEGF is restricted to vascular endothelial cells. Increased expression of VEGF has been described recently in the hyperthecotic stroma of polycystic ovaries (PCO; Kamat *et al.*, 1995). Increased ovarian stromal blood flow in women with PCO has been demonstrated previously by colour Doppler blood flow imaging (Battaglia *et al.*, 1995; Zaidi *et al.*, 1995b; Aleem and Predanic, 1996). VEGF may also be a factor responsible for maintaining perifollicular blood flow and regulation of intrafollicular oxygen levels (Van Blerkom *et al.*, 1997).

The aim of this study was to determine serum VEGF concentrations and Doppler blood flow changes within the ovarian stroma of women with PCO and women with normal ovaries. We aimed to establish whether there is a relationship between serum VEGF concentrations and Doppler blood flow velocities within the ovarian stroma of women with normal and polycystic ovaries.

Materials and methods

Subjects

We recruited 60 consecutive women (46 nulliparous and 14 multiparous) who attended the London Women's Clinic for in-vitro fertilization (IVF) treatment. These women had no concomitant pelvic pathology, such as endometriosis, uterine fibroids or ovarian cysts. The 60 women were divided into three groups according to the following criteria. The 'normal ovary group' ($n = 36$) had regular ovulatory menstrual cycles and normal ovaries, as demonstrated on baseline ultrasound examination. The 'PCO group' ($n = 14$) had regular ovulatory menstrual cycles and PCO on pretreatment ultrasonographic assessment of the ovaries on days 2–3 of the menstrual cycle on at least one or on several occasions if the patient had been treated previously in the clinic (Adams *et al.*, 1985; Conway *et al.*,

Table I. Demographic data of the three groups of women

Variables	Normal ovaries (n = 36)	Polycystic ovaries (n = 14)	Polycystic ovarian syndrome (n = 10)
Mean age (years; range)	36.2 (28–43)	35.2 (25–40)	33.1 (28–41)
Mean ± SD body mass index (kg/m^2)	22.9 ± 2.8	24.0 ± 4.4	26.9 ± 2.2[a]
No. of parous women (%)	5 (13.8)	5 (35.7)	4 (40.0)
Duration of infertility (years)	6.8 ± 3.1	7.0 ± 3.8	6.7 ± 2.7
Causes of infertility			
Male factor (MF)	10 (27.7%)	5 (35.7%)	3 (30%)
Tubal factor (TF)	6 (16.6%)	3 (21.4%)	2 (20%)
Mixed (MF + TF)	5 (13.8%)	0	0
Endometriosis	7 (19.4%)	3 (21.4%)	2 (20%)
Unexplained	8 (22.2%)	3 (21.4%)	3 (30%)

[a]Statistically significant difference (P = 0.0001) between women with normal ovaries and polycystic ovaries compared with women with polycystic ovarian syndrome.

Table II. Serum vascular endothelial growth factor (VEGF) and hormone concentrations in the three groups of women

Hormones (mean ± SD)	Normal ovaries (n = 36)	Polycystic ovaries (n = 14)	Polycystic ovarian syndrome (n = 10)	Statistical significance
VEGF (ng/ml)	2.30 ± 0.55	3.4 ± 0.7[a]	3.20 ± 0.66[b]	<0.0001
Follicle stimulating hormone (IU/l)	6.8 ± 4.4	6.1 ± 1.4	5.9 ± 2.2	NS
Luteinizing hormone (IU/l)	4.5 ± 2.2	5.4 ± 2.4	9.2 ± 2.8[b]	<0.0001
Oestradiol (pmol/l)	158.3 ± 96.0	105 ± 45	134.6 ± 61.0	NS
Testosterone (nmol/l)	0.83 ± 0.50	0.88 ± 0.30	1.5 ± 1.2[b]	<0.05

NS = not significant.
[a]Statistically significant differences between women with normal ovaries and women with polycystic ovaries and polycystic ovary syndrome.
[b]Statistically significant differences between women with normal ovaries and women with polycystic ovarian syndrome.

1989; Fox *et al.*, 1991; Balen *et al.*, 1995) but did not have clinical or biochemical evidence of polycystic ovarian syndrome (PCOS). The 'PCOS group' (n = 10) had PCO on ultrasound examination and a history of anovulatory menstrual cycles and/or oligomenorrhoea, with or without hirsutism, acne and obesity and/or elevated serum LH (>10 IU/l) and/or elevated serum androgen concentrations. The descriptive data of the three groups of women are presented in Tables I and II.

Pelvic ultrasonography and Doppler blood flow velocity measurements

Ultrasound examinations were performed using a 5 MHz transvaginal transducer with colour and pulsed Doppler facilities (128XP/10 OB; Acuson, Mountain View, CA, USA). All examinations were performed at the beginning of a menstrual cycle (day 2 or 3) prior to starting IVF treatment.

The spatial peak temporal average intensity of ultrasound for B-mode and Doppler examinations was <50 mW/cm^2. Colour flow mapping and pulsed Doppler measurements were performed on ovarian stromal blood vessels once normal pelvic findings were confirmed. Areas of maximum colour intensity, representing the greatest Doppler frequency shifts, were selected for pulsed Doppler examination. Blood flow velocity waveforms were thus detected and recorded. The peak systolic blood flow velocity (PSV), time-averaged maximal velocity (TAMXV), pulsatility index (PI) and resistance index (RI) were assessed within both ovaries. PI values for each vessel were calculated from smooth curves fitted to the waveforms over three cardiac cycles according to the formula PI = $(S - D)$/TAMXV, where S is the peak systolic velocity, D is the minimum Doppler shifted frequency over a cardiac cycle and TAMXV is the time-averaged maximum velocity over a cardiac cycle (Gosling *et al.*, 1971). The PI and RI were used as measures of blood flow impedance distal to the point of sampling.

All observations were made by R.A., P.S. and L.E. The interobserver coefficient of variation (CV) for TAMXV was 24% and for PI was 14%, as described previously (Sladkevicius *et al.*, 1993). For TAMXV and PI, the intra-observer CV was <10%. All examinations were performed before midday to reduce the effects of diurnal variations in blood flow (Zaidi *et al.*, 1995a). Blood flow images were recorded on video and stored for later analysis.

VEGF assay

Blood samples for serum VEGF and hormone measurements were obtained between 08:00 and 12:00 h immediately after the ultrasound examination. Serum was stored at –70°C. VEGF concentrations were measured using an enzyme immunoassay (Cytokit Red™ EIA kits; Peninsula Labs Inc., College Park, MD, USA). The assay sensitivity was 0.195 ng/ml. The detectable range was 0.195–200 ng/ml, and cross-reactivity was <0.5% against cytokine standards (intra-assay CV 7.8%, inter-assay CV 12.2%).

Hormone assays

Follicle stimulating hormone (FSH) and LH were measured by a microparticle enzyme immunoassay (Abbot AxSYM reagent pack; Abbot, IL, USA). Oestradiol and testosterone concentrations were assessed using radioimmunoassays (Sorin clinical assays and coated

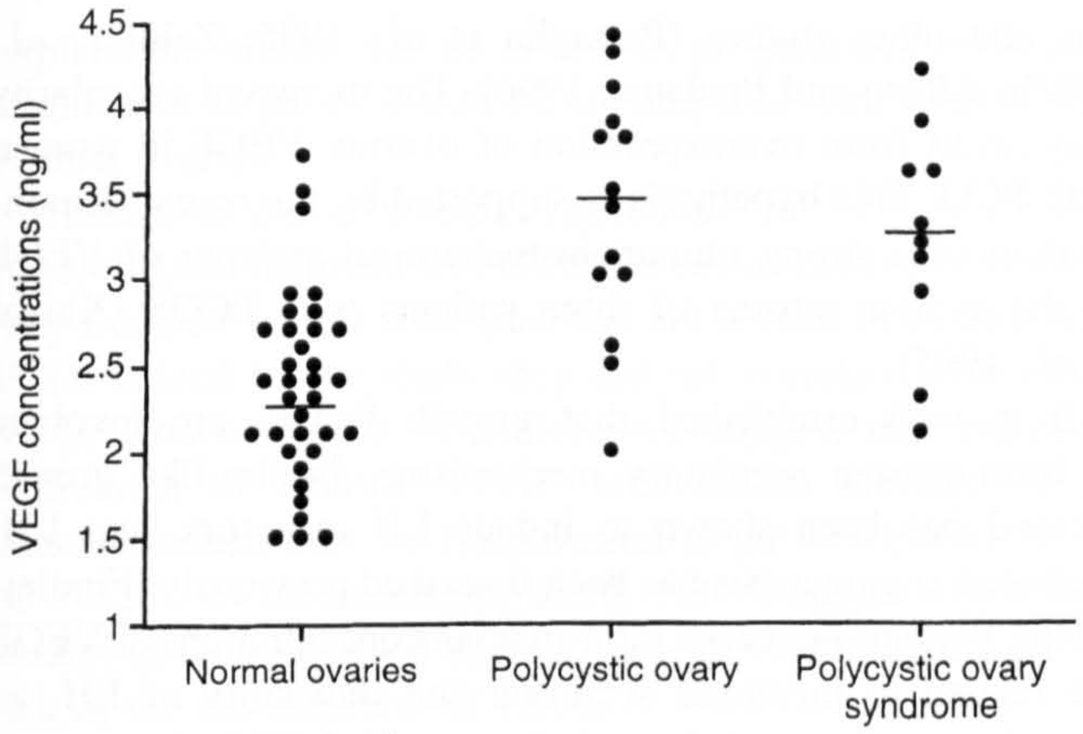

Figure 1. Serum vascular endothelial growth factor (VEGF) concentrations were higher in women with polycystic ovaries (3.4 ± 0.7 ng/ml) and polycystic ovarian syndrome (3.20 ± 0.66 ng/ml) than in women with normal ovaries (2.3 ± 0.5 ng/ml) ($P < 0.0001$).

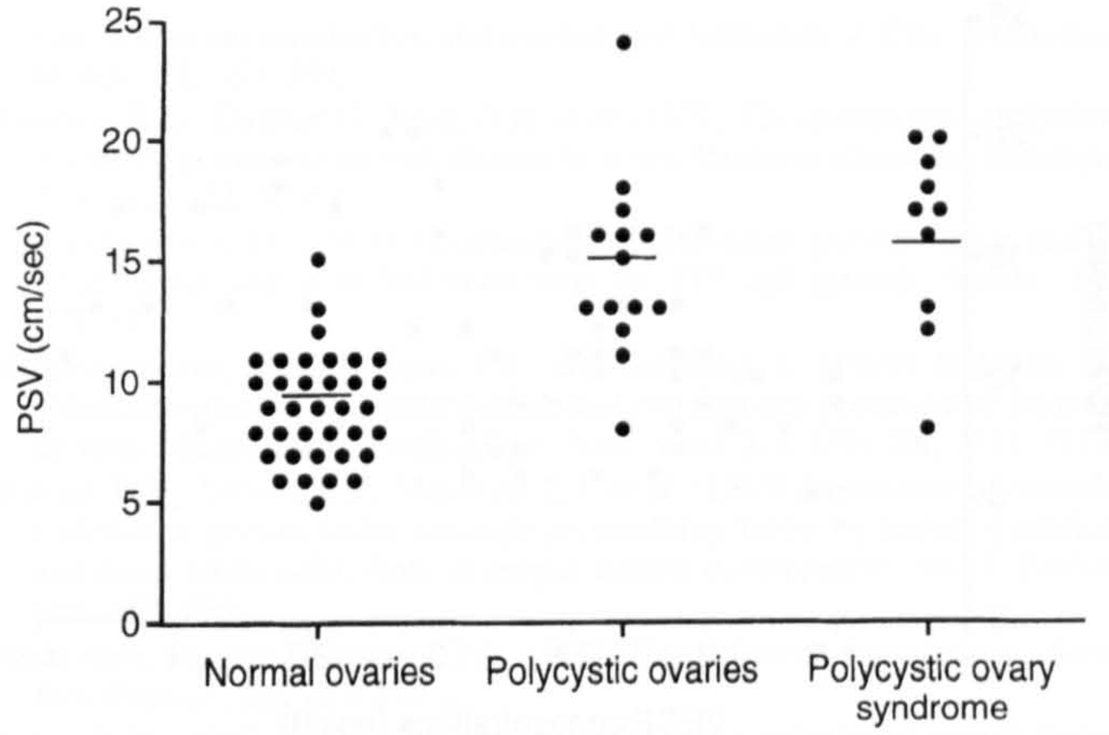

Figure 2. Peak systolic velocity (PSV) was higher in women with polycystic ovaries (15 ± 4 cm/s) and polycystic ovarian syndrome (16 ± 4 cm/s) than in women with normal ovaries (9 ± 2 cm/s) ($P < 0.001$).

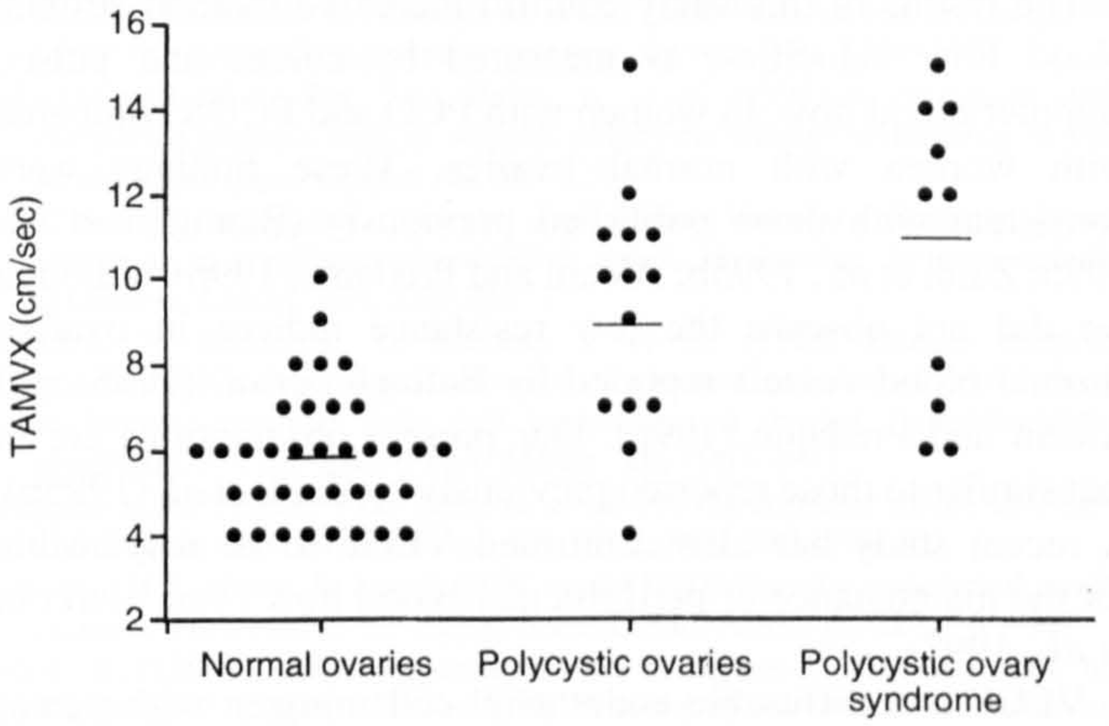

Figure 3. Time-averaged maximum velocity (TAMXV) was higher in women with polycystic ovaries (9 ± 3 cm/s) and polycystic ovarian syndrome (11 ± 3 cm/s) than in women with normal ovaries (5.8 ± 1.5 cm/s) ($P < 0.001$).

tubes; DPC, Los Angeles, CA, USA). The intra- and interassay CV were 4.0 and 7.5% for FSH, 4.0 and 7.5% for LH, 6.0 and 7.5% for oestradiol and 6.2 and 8.0% for testosterone respectively.

Statistical analysis

Data are represented as means ± SD. Comparisons between the three groups were performed using an analysis of variance. Student's *t*-test was used to compare continuous variables. P values < 0.05 were considered to be statistically significant. Correlations between variables were sought using Pearson's correlation coefficient.

Results

Demographic data from the three study groups were similar. However women with PCOS had a significantly higher body mass index ($P < 0.001$) than women with normal ovaries and PCO (Table I).

Serum VEGF and hormone concentrations of the three groups of women are shown in Table II. Mean serum VEGF concentrations in women with PCO and PCOS were significantly higher ($P < 0.0001$) than in women with normal ovaries (Figure 1). There were no statistically significant differences in serum VEGF concentrations between women with PCO and those with PCOS. Significantly higher LH and testosterone concentrations were found in women with PCOS than in the other two groups (Table II).

Recordings of blood flow velocity waveforms from the ovarian stroma were possible in 98.4% of women with PCO and in 88.4% in women with normal ovaries. Analyses of Doppler blood flow velocities of ovarian stromal blood vessels showed no significant differences in the PSV, TAMXV, PI or RI values between the right and left ovaries. Mean values of the two blood flow velocity measurements were therefore calculated and used for subsequent analyses. A subjective assessment of the ovaries demonstrated a larger number of blood vessels and a greater intensity of colour blood flow within the ovarian stroma of women with PCO and PCOS compared with women with normal ovaries.

PSV and TAMXV values were significantly greater in women with PCO and PCOS than in women with normal ovaries (Figures 2 and 3). There were no significant differences in the blood flow velocities (PSV and TAMXV) of women with PCO compared with women with PCOS. Mean RI and PI values were not different between the three groups of patients.

A positive correlation was demonstrated between serum VEGF concentrations and PSV ($r = +0.41$, $P = 0.001$) and TAMXV ($r = +0.45$, $P < 0.001$) in women in all three groups (Figure 4). No correlations were observed between VEGF concentrations and serum FSH, LH, oestradiol and testosterone concentrations. No relationship was found between serum hormone concentrations and peak blood flow velocities measured on day 2 or 3 of the menstrual cycle.

Discussion

The important findings of this study are that women with PCO have elevated serum VEGF concentrations and higher ovarian stromal blood flow velocities than women with normal ovaries. We also found a positive correlation between serum VEGF concentrations and blood flow velocities (PSV and TAMXV).

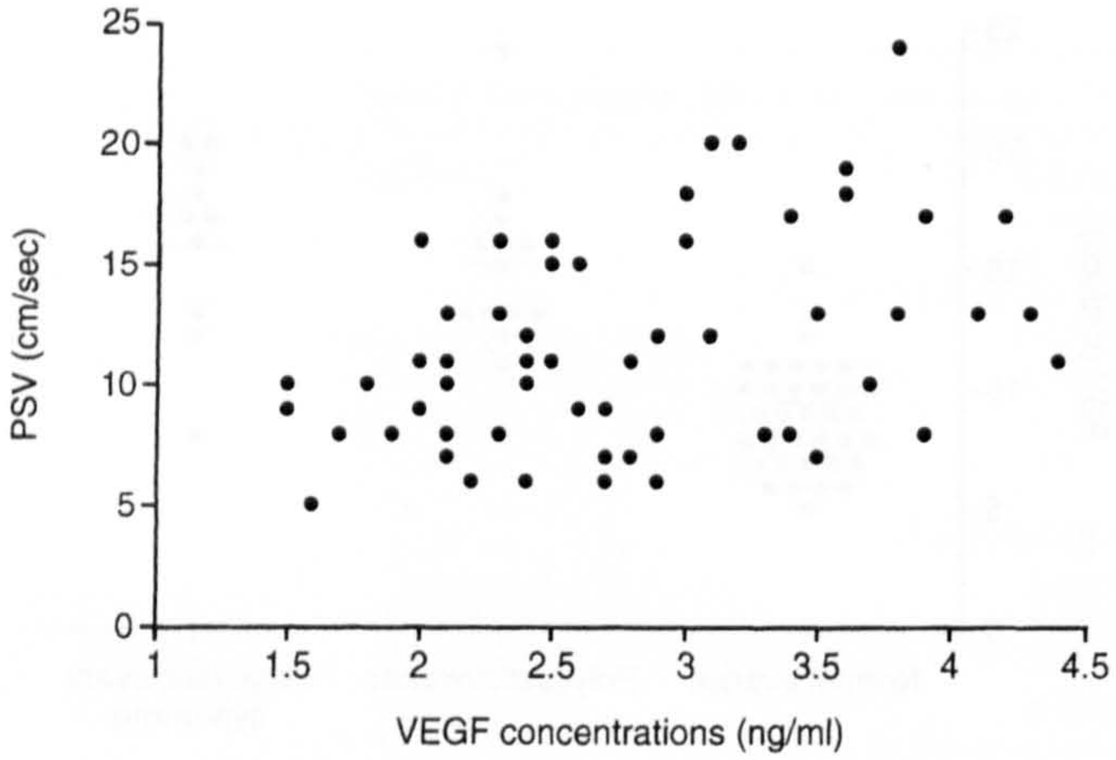

Figure 4. A positive correlation was observed between serum vascular endothelial growth factor (VEGF) concentrations and peak systolic velocity (PSV; $r = +0.44$, $P < 0.001$).

The results of this study confirm increased ovarian stromal blood flow velocities, as measured by colour and pulsed Doppler blood flow, in women with PCO and PCOS compared with women with normal ovaries. These findings were consistent with those published previously (Battaglia *et al.*, 1995; Zaidi *et al.*, 1995b; Aleem and Predanic, 1996), although we did not observe the low resistance indices in ovarian stromal blood vessels reported by Battaglia *et al.* (1995) and Aleem and Predanic (1996). Our present observations are in fact similar to those reported previously by Zaidi *et al.* (1995b). A recent study has also confirmed VEGF to be responsible for the maintenance of perifollicular blood flow (Van Blerkom *et al.*, 1997).

VEGF is a diffusable endothelial cell mitogen with potent angiogenic properties (Senger *et al.*, 1983; Ferrara and Henzel, 1989; Gospodarowicz *et al.*, 1989; Leung *et al.*, 1989; Connolly, 1991; Dvorak *et al.*, 1995). It mediates neovascularization in various biological processes, e.g. corpus luteum formation, embryogenesis, tumorigenesis and wound healing (Ferrara and Davis-Smith, 1997). VEGF mRNA is expressed in cells surrounding expanding vasculature. It is also constitutionally expressed in healthy human beings but, less so, in adults compared with fetuses, where it may play an important role in neoangiogenesis and organogenesis. VEGF is expressed more in pathological conditions, including tumours. Within the ovary it has been demonstrated in theca cells (Gordon *et al.*, 1996), granulosa and lutein cells (Phillips *et al.*, 1990; Shweiki *et al.*, 1993; Koos, 1995; Neulen *et al.*, 1995) and the interstitial tissue (Kamat *et al.*, 1995). VEGF not only mediates angiogenesis but also induces connective tissue stromal growth by increasing microvascular permeability, which leads to extravasation of plasma proteins. The extravascular matrix thus formed favours in-growth of new blood vessels and fibroblasts, which in turn organizes the avascular provisional fibrin matrix into a mature, vascularized connective tissue stroma (Kamat *et al.*, 1995).

These observations and our findings of elevated serum VEGF concentrations in women with PCO may explain in part the dense hyperechogenic and highly vascularized stroma of PCO, as demonstrated by Doppler blood flow studies in this and other studies (Battaglia *et al.*, 1995; Zaidi *et al.*, 1995b; Aleem and Predanic, 1996). The increased vascularity may result from overexpression of ovarian VEGF in women with PCO. This hypothesis is supported by the recent demonstration of a strong immunohistochemical staining of VEGF in the ovarian stroma of three patients with PCOS (Kamat *et al.*, 1995).

It is well established that growth factors are involved in intra-ovarian regulatory mechanisms. Insulin-like growth factor-I has been shown to induce LH receptors, and LH-mediated angiogenesis has been described previously (Findlay, 1986). Perhaps increased intra-ovarian concentrations of VEGF are related to increased secretion and pulsatility of LH, an important pathophysiological feature of PCOS. There are, however, large fluctuations of LH concentration in women with PCOS over time (Adashi *et al.*, 1995). Moreover, raised LH concentrations are seen in only 40% of women with PCOS (Conway *et al.*, 1989; Balen *et al.*, 1995). In addition, our study has shown that elevated concentrations of VEGF and increased stromal blood flow are found in women with PCO as well as those with PCOS, consistent with it being a constitutive feature of PCO. Unlike other growth factors responsible for angiogenesis, e.g. bFGF, which is largely intracellular and non-diffusable, VEGF is a soluble, diffusable growth factor. There is also evidence to suggest that angiogenic factors, like bFGF, TGF-β, platelet-derived growth factor and nitric oxide, act as agonists to the action of VEGF (Connolly, 1991).

Oestradiol plays an important role as a moderator of uterine and ovarian vascularity (Steer *et al.*, 1990; de Ziegler *et al.*, 1991). However, visualization of distinct stromal blood vessels in PCO in the early follicular phase of the menstrual cycle is the first striking difference from normal ovaries in the vascular pattern. We consider that this difference is unlikely to be caused by oestradiol, particularly because its concentrations are not raised in women with PCO.

It is well known that the risk of ovarian hyperstimulation syndrome (OHSS) in ovulation induction and in-vitro fertilization programmes is higher in women with PCO than women with normal ovaries (Rizk, 1991; MacDougall *et al.*, 1993). Again this finding might be the result of overexpression of VEGF in women with PCO, who characteristically recruit excess numbers of follicles with even small doses of gonadotrophin stimulation. Elevated concentrations of VEGF in various body fluids, e.g. ascitic fluid, follicular fluid, serum and urine, have been established recently in women undergoing ovarian stimulation who develop OHSS (McClure *et al.*, 1994; Robertson *et al.*, 1995; Krasnow *et al.*, 1996; Abramov *et al.*, 1997; Elchalal and Schenker, 1997; Rizk *et al.*, 1997). Our current findings provide a mechanism that helps to explain the link between VEGF, OHSS and PCO.

In conclusion, in women with PCO the increased vascularity that underlies the increased blood flow demonstrated by Doppler blood flow velocity measurements may be related to the higher serum concentrations of VEGF. In addition, VEGF also increases vascular permeability, which may contribute to the formation of the increased stroma in PCO and the increased capillary leakage associated with OHSS. The results may

explain the higher risk of OHSS in programmes of ovarian stimulation in patients with PCO compared with women with normal ovaries.

Though an elevated serum concentration of LH is a pathophysiological hallmark of PCOS, values of LH concentrations may fluctuate widely over time and are normal in women with PCO; indeed in our study they did not correlate with serum VEGF concentrations. Therefore we suggest that increased expression of ovarian VEGF, reflected in elevated serum concentrations, may be fundamental to the aetiopathogenesis of PCOS. Its measurement, along with Doppler blood flow studies of ovarian blood vessels, may provide an index for the risk of the development of OHSS.

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Serum vascular endothelial growth factor (VEGF) in the normal menstrual cycle: association with changes in ovarian and uterine Doppler blood flow

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Summary

OBJECTIVE To investigate whether changes in circulating serum vascular endothelial growth factor (VEGF) concentrations during the menstrual cycle are associated with changes in blood flow within the ovaries and uterus.

PATIENTS AND MEASUREMENTS Serum VEGF concentrations were measured and pulsed and colour Doppler blood flow waveforms recorded within the ovarian stroma and uterine arteries during the early follicular, the immediate preovulatory and the mid-luteal phases of the menstrual cycle of 14 healthy women.

RESULTS Mean (±SD) serum VEGF concentrations rose from 2·44 ± 0·1 ng/ml in the early follicular phase to 3 ± 0·8 ng/ml in the pre-ovulatory phase and to 4·4 ± 0·9 ng/ml in the mid-luteal phase ($P<0·0001$) of the menstrual cycle. Mean peak systolic blood flow velocity (PSV) and time-averaged maximum flow velocity (TAMXV) were higher within the ovarian stroma of the ovary bearing the dominant follicle and the uterine arteries in the pre-ovulatory and mid-luteal phase than in the same sites during the early follicular phase of the menstrual cycle. PSV rose significantly from the early follicular phase (11 ± 6 cm/s) to 14 ± 4 cm/s in the pre-ovulatory phase and further in the mid-luteal phase (26 ± 7 cm/s, $P=0·0001$). Within the uterine arteries, mean PSV rose significantly from 28 ± 9 cm/s in the early follicular phase to 31 ± 8 cm/s in the pre-ovulatory phase and further in the mid-luteal phase (44 ± 11 cm/s, $P<0·005$). Serum VEGF correlated with serum progesterone concentrations in the luteal phase ($r=0·85$, $P<0·001$), with serum oestradiol concentrations in the early follicular ($r=0·67$, $P=0·009$), pre-ovulatory ($r=0·57$, $P=0·03$) and luteal phases ($r=0·68$, $P<0·005$) and with serum testosterone in the early follicular phase ($r=0·63$, $P=0·01$).

CONCLUSIONS Cyclical changes in serum vascular endothelial growth factor concentrations are associated with coincident changes in ovarian and uterine blood flow.

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While changes in uterine and ovarian vascularity during the normal menstrual cycle, as demonstrated by pulsed and colour Doppler blood flow measurements, have been described (Scholtes *et al.*, 1989; Steer *et al.*, 1990; Sladkevicius *et al.*, 1993) the factors which control them are not fully understood.

Following exposure of the dominant ovarian follicle to the mid-cycle surge of luteinizing hormone (LH), or to an injection of human chorionic gonadotrophin (hCG), blood vessels grow from the theca into the recently ruptured follicle to form a complex vascular network, a major feature of the development and differentiation of the corpus luteum. Numerous angiogenic factors have been implicated in corpus luteal and uterine angiogenesis. Vascular endothelial growth factor (VEGF), a recently identified diffusible angiogenic factor, is emerging as an important factor for neovascularization of the reproductive tract (Gospodarowicz *et al.*, 1989, Leung *et al.*, 1989, Connolly, 1991, Ferrara & Davis-Smyth, 1997).

The aim of the present study was to determine whether changes in peripheral VEGF concentrations during the menstrual cycle reflect changes in Doppler blood flow velocities within the ovarian stroma and the uterine arteries.

Materials and methods

Subjects

We recruited 19 healthy volunteers with regular ovulatory

menstrual cycles (range 28–30 days, previous ovulation confirmed by measurements of serum progesterone in excess of 30 nmol/l) who were not receiving treatment with the oral contraceptive pill. Those with a history or examination suggestive of endometriosis ($n=2$), uterine fibroids ($n=2$) or recent pelvic inflammatory disease ($n=1$) were excluded. The study group then comprised 14 women, 10 of whom were nulliparous. Ten women had normal ovaries and four had polycystic ovaries (PCO), as determined by ultrasonographic criteria (Adams *et al.*, 1985; Fox *et al.*, 1991). The mean age was 25 (range 23–31) years and mean ± SD body mass index (BMI) was 23·6 ± 2·8 kg/m^2.

Pelvic ultrasonography and Doppler ultrasound blood flow velocity measurements

Ultrasound examination was performed via the transvaginal route using a 5 MHz transducer with colour and pulsed Doppler facilities (Acuson computed sonography (128XP/10 OB; Mountain View, CA, USA), as described previously (Sladkevicius *et al.*, 1993; Zaidi *et al.*, 1995). Baseline measurements of peak systolic velocity (PSV) and time averaged maximum velocity (TAMXV), (measurements of blood flow velocity) and the pulsatility and resistance indices (PI and RI) (measurements of the resistance of blood flow) were made both on ovarian stromal blood vessels and on the uterine arteries at the beginning of the menstrual cycle (days 2 or 3).

Daily ultrasound examinations were performed from the 11th day of the menstrual cycle for tracking the growth of the dominant follicle. Further measurements of Doppler ultrasound blood flow were performed once the dominant follicle reached 18–20 mm in diameter. A blood sample was obtained to measure serum concentrations of LH and VEGF on this day. Twelve subjects had a LH surge on the day of the examination and two the following day (confirmed by urinary LH surge). Doppler blood flow, serum VEGF and hormone measurements were repeated again 7 days after ovulation. As observed by vaginal ultrasound examination, each woman developed a dominant follicle and subsequently a corpus luteum, accompanied by a mid-luteal serum progesterone concentration in excess of 30 nmol/l. Mean values of the serum hormone concentrations during the three phases of the menstrual cycle are showed in Table 1.

A single observer (RA) made all observations. The intra-observer coefficient of variation was less than 10% for TAMXV and PI (data not shown). All examinations were performed before midday to reduce the effects of diurnal variation in blood flow (Zaidi *et al.*, 1995). Blood flow images were recorded on video tape and stored for later analysis.

VEGF assay

Blood samples for serum VEGF and hormone measurements were obtained between 0800 h and 1200 h immediately after the ultrasound examination. Serum was stored at −70°C for subsequent analysis. VEGF concentrations were measured using enzyme immunoassay (Cytokit Red™ EIA kits, Peninsula Labs, Inc., College Park, MD, USA). The assay sensitivity was 0·2 ng/ml (intra-assay coefficient of variation 7·8%, inter-assay coefficient of variation 12·2%). The cross-reactivities of the assay were <0·5% against WHO cytokine standards.

VEGF measured was the 'total' circulating VEGF bound to plasma proteins (e.g. α_2 macroglobulin, Houck *et al.*, 1992). The major circulating forms of VEGF are $VEGF_{121}$ and $VEGF_{165}$, while $VEGF_{189}$ and $VEGF_{206}$ are largely bound to cell membranes and do not represent measurable VEGF (Houck *et al.*, 1992, Soker *et al.*, 1993, Heney *et al.*, 1995, Anthony *et al.*, 1997).

Hormone assays

Follicle stimulating hormone (FSH) and luteinizing hormone were measured by microparticle enzyme immunoassay (Abbot AxSYM reagent pack, IL, USA) and oestradiol, progesterone and testosterone by chemiluminescent assays (Immulite kits DPC, Los Angeles, CA, USA). The intra- and inter-assay coefficients of variation were 2·9 and 3·7% for FSH, 1·8 and

Table 1 Mean ± SD values of serum hormone measurements during the menstrual cycle

Hormone value	Early follicular phase	Pre-ovulatory phase	Mid-luteal phase
FSH (iu/l)	4·57 ± 1	11·5 ± 5·2	ND
LH (iu/l)	4 ± 1·4	38·4 ± 9·8	ND
Oestradiol (pmol/l)	123·2 ± 44	779 ± 371	334 ± 173
Testosterone (nmol/l)	0·81 ± 0·5	ND	ND
Progesterone (nmol/l)	ND	ND	42·7 ± 18·3

ND: not measured.

4·1% for LH, 9·5 and 15% for oestradiol, 8·6% and 12% for progesterone and 6·6 and 9·2% for testosterone.

Statistical analysis

Statistical analyses were performed using SPSS for Windows. Data are represented as mean ± SD. Comparisons between the three phases of the menstrual cycle were performed by analysis of variance (ANOVA). *Post hoc* analysis was undertaken using Scheffé's test. Power calculation of the study was performed after analysis of results from six women. The analysis showed that 11 patients were necessary to show a 90% reliability of results. Correlations between variables were sought by Pearson's correlation coefficient. Covariate analysis was performed using general factorial ANOVA. P values of $<0{\cdot}05$ were considered significant.

Results

Mean serum VEGF concentrations (Fig. 1) were higher in the mid-luteal phase (4·43 ± 0·9 ng/ml) than in the early follicular (2·44 ± 0·2 ng/ml) and immediate pre-ovulatory phase (3 ± 0·8 ng/ml, $P<0{\cdot}0001$). Women with polycystic ovaries had higher serum VEGF concentrations in the early follicular phase than in women with normal ovaries (3·1 ± 0·2 ng/ml *vs.* 1·6 ± 0·2 ng/ml) (Fig. 1).

Analyses of Doppler blood flow velocities in the ovarian stroma showed no significant differences in the PSV, TAMXV, PI or RI between the right and left ovaries during the baseline (early follicular phase) scan. Differences were noted however, during the pre-ovulatory and mid-luteal phases. Subjective assessment of the ultrasound scan of the ovaries during the mid-luteal phase indicated a larger number of blood vessels and a greater intensity of colour blood flow within the ovarian stroma of the dominant ovary (i.e. the side of ovulation) compared with the non-dominant ovary.

Analyses of Doppler blood flow velocities of the uterine arteries showed no significant differences in the PSV, TAMXV, PI or RI between the right and left uterine arteries (i.e. between the sides of the dominant and non-dominant ovary). Mean values for the two blood flow velocity and resistance measurements were therefore calculated and used for subsequent analyses.

In the ovary containing the dominant follicle, mean PSV was higher in the pre-ovulatory and the mid-luteal phases compared with the early follicular phase (Fig. 2). Mean TAMXV was also higher in the pre-ovulatory phase (10 ± 5 cm/s, day of LH surge) and the mid-luteal phase (17 ± 4 cm/s) compared with the early follicular phase (6 ± 2 cm/s, $P<0{\cdot}0001$). The PI decreased significantly in the pre-ovulatory and the mid-luteal phases (0·78 ± 0·18 and 0·69 ± 0·14) compared with the early follicular phase (0·96 ± 0·2, $P=0{\cdot}001$) although no changes in the RI were detected. No cyclical changes in blood flow velocities in the non-dominant ovary were detected.

In the uterine arteries, mean PSV was higher in the pre-ovulatory and mid-luteal phases compared with the early follicular phase (Fig. 3). Mean TAMXV was also higher in the pre-ovulatory phase (8 ± 3 cm/s) and in the mid-luteal phase (14 ± 3 cm/s) compared with the early follicular (7 ± 2 cm/s, $P<0{\cdot}0001$). The PI was significantly lower in the pre-ovulatory and the mid-luteal phases (2·63 ± 0·67 and 2·15 ± 0·54) compared with the early follicular phase (3·14 ± 0·6, $P<0{\cdot}005$). Although the RI decreased, these changes did not reach statistical significance.

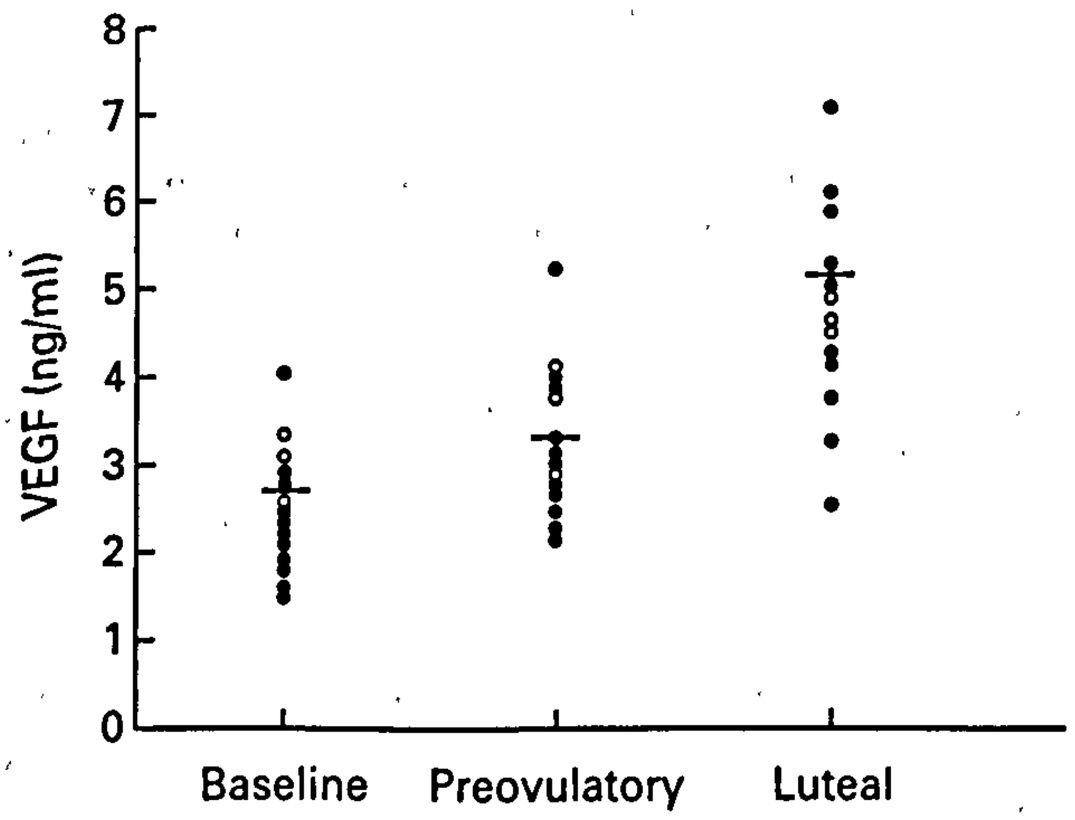

Fig. 1 Serum VEGF concentrations were higher in the mid-luteal phase ($P<0{\cdot}0001$) compared with those in early follicular and pre-ovulatory phases. ●, VEGF concentrations in women with normal ovaries; ○, VEGF concentrations in women with polycystic ovaries.

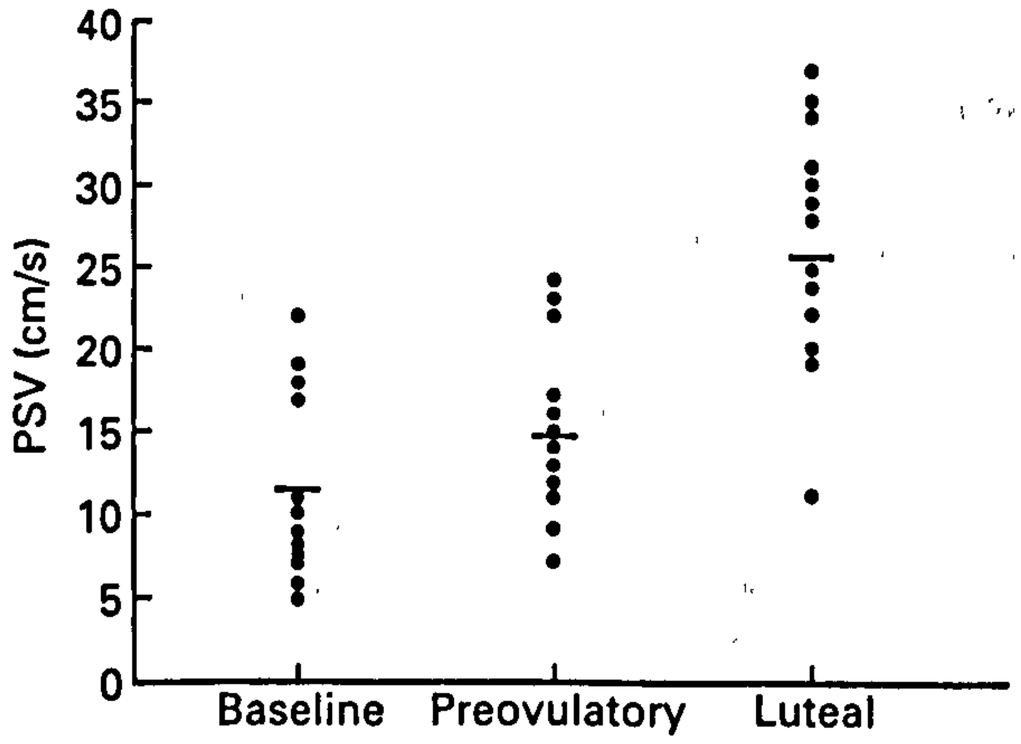

Fig. 2 Ovarian stromal peak systolic velocities (PSV) were higher ($P<0{\cdot}0001$) in the mid-luteal phase compared with those in early follicular and pre-ovulatory phases.

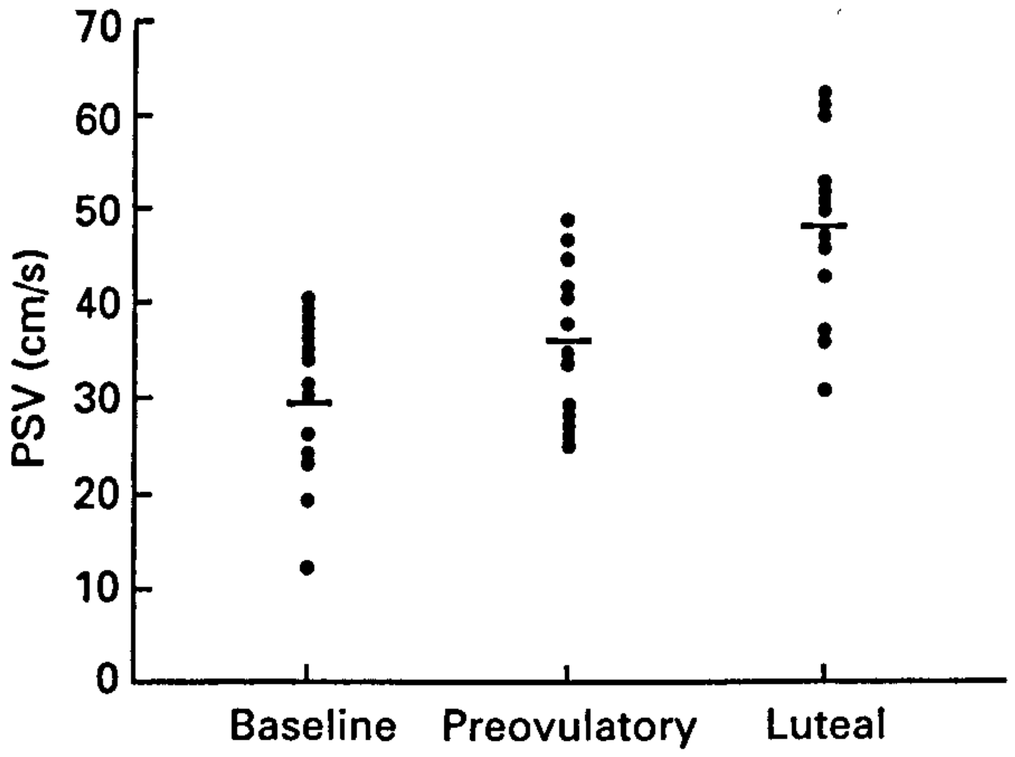

Fig. 3 Uterine artery peak systolic velocities (PSV) were higher ($P<0{\cdot}0007$) in the mid-luteal phase compared with those in early follicular phase and the pre-ovulatory phase.

Postive correlations were observed between serum VEGF and progesterone measurements in the mid-luteal phase ($r=0{\cdot}85$, $P<0{\cdot}0001$), between serum VEGF and oestradiol concentrations in early follicular ($r=0{\cdot}66$, $P<0{\cdot}01$), pre-ovulatory ($r=0{\cdot}57$, $P=0{\cdot}03$) and mid-luteal phases ($r=0{\cdot}68$, $P<0{\cdot}005$) and between serum VEGF and testosterone concentrations in the early follicular phase of the menstrual cycle ($r=0{\cdot}63$, $P=0{\cdot}01$). No statistically significant association of VEGF measurements with serum concentrations of LH and FSH were detected.

The close relationship of serum VEGF with uterine and dominant ovarian blood flow velocities is demonstrated in Figs 1–3. Using covariate analysis, the changes in serum VEGF concentrations during the menstrual cycle remained significant ($P<0{\cdot}0001$, ANOVA), even after controlling peak systolic blood flow velocities in both ovarian and uterine arteries ($P=0{\cdot}048$).

Discussion

We found that serum VEGF concentrations rose in the luteal phase of the menstrual cycle in parallel with changes in ovarian and uterine blood flow velocities. Although there was a close relationship between serum VEGF measurements and blood flow velocities (Figs 1–3) the causal relationship between the factors is a matter for speculation. The fact that the rise in serum VEGF concentrations during the menstrual cycle remained significant after controlling statistically for changes in ovarian and uterine artery blood flow suggests that changes in VEGF, reflected in its serum concentrations, is one of the primary events that causes vascular growth.

VEGF is produced by the ovary immediately before (Ravindranath *et al.*, 1992; Shweiki *et al.*, 1993; Koos *et al.*, 1995; Gordon *et al.*, 1996) and after ovulation and is thought to mediate neovascularization of the corpus luteum (Phillips *et al.*, 1990; Yan *et al.*, 1993; Koos *et al.*, 1995; Kamet *et al.*, 1995). We found that these dynamic changes within the ovary were reflected in the peripheral venous concentrations of VEGF. Changes in VEGF during the normal menstrual cycle have also been demonstrated in histological sections of the human endometrium (Shifren *et al.*, 1996). Further, we observed positive correlations between serum VEGF and progesterone concentrations in the mid-luteal phase of the menstrual cycle. Higher basal serum VEGF concentrations were found in women with polycystic ovaries compared with women with normal ovaries, consistent with our earlier report on a larger and different group of subjects (Agrawal *et al.*, 1998). The rise in serum VEGF concentrations in the luteal phase of the menstrual cycle would be consistent with the reproductive tract making a major contribution to circulating VEGF concentrations. Although the expression of VEGF messenger RNA is temporally and spatially related to the proliferation of blood vessels in spontaneously cycling and in hormonally induced ovulation of all species, other molecules such as bFGF, angiopoetin 1, 2 and PIGF are equally implicated in corpus luteal angiogenesis. However, recent work by Ferrara and colleagues has shown complete inhibition of VEGF bioactivity, suppression of corpus luteal formation and progesterone production by using soluble human and murine tyrosine kinase receptor fixed Fc immunoglobulin in a murine ovary where ovulation was induced with gonadotrophins and hCG. This indicates that VEGF is essential for corpus luteal angiogenesis (Ferrara *et al.*, 1998).

Although adult organs (e.g. liver, lung, breast and carcinomas) do express VEGF mRNA, we can find no evidence to suggest that they contribute to serum VEGF concentrations, cyclically or acyclically (Ferrara & Davis-Smyth, 1997).

The Doppler blood flow findings we report here are consistent with those published previously (Goswamy *et al.*, 1988; Steer *et al.*, 1990; Battaglia *et al.*, 1990; Santolaya Forgas, 1992; Sladkevicius *et al.*, 1993; Campbell *et al.*, 1993; Bourne *et al.*, 1996; Lunenfeld *et al.*, 1996).

VEGF is also expressed throughout the smooth muscle of the uterus. VEGF gene expression within the uterus is rapidly stimulated by oestradiol. It seems likely, therefore, that VEGF mediates the oestrogen-induced increase in uterine vascular permeability and growth (Charnock-Jones *et al.*, 1993; Cullinan-Bove & Koos, 1993; Hyder *et al.*, 1996). The positive correlation of serum VEGF and oestradiol reported here is consistent with this finding. VEGF mRNA in normal myometrium and endometrium are significantly higher in the secretory phase than in the proliferative phase of the menstrual cycle (Li *et al.*, 1994; Harrison-Woolrych *et al.*, 1995; Torry *et al.*, 1996). VEGF may be the principal factor that promotes the vascular

growth, maintenance and hyperpermeability required for adequate receptivity of the cycling human endometrium. A positive correlation was also observed between serum VEGF and testosterone concentrations but not LH concentrations. This suggests that testosterone and not LH may upregulate VEGF gene expression. There is also evidence to support the suggestion that testosterone upregulates VEGF expression within the prostate gland after castration (Franck-Lissbrant *et al.*, 1998).

In conclusion, our study establishes a possible link between serum vascular endothelial growth factor and cyclical changes within the uterus and the dominant ovary, as demonstrated by their correlation with changing Doppler blood flow velocities during the normal menstrual cycle and a positive correlation with serum progesterone and oestradiol concentrations in the mid-luteal phase of the menstrual cycle. Further studies are in progress to determine the extent to which peripheral venous concentrations of vascular endothelial growth factor arise from the ovary and the uterus.

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Serum vascular endothelial growth factor concentrations in postmenopausal women: the effect of hormone replacement therapy

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Objective: To assess serum vascular endothelial growth factor (VEGF) concentrations in healthy postmenopausal women in relation to hormone replacement therapy (HRT) and the presence or absence of a uterus.

Design: Cross-sectional study.

Setting: The Middlesex Hospital.

Patient(s): A total of 199 postmenopausal women were enrolled: 132 had uterus in situ and 67 had had hysterectomies. Of the 67 women who had had hysterectomies, 6 received no HRT, 20 received tibolone, 25 received transdermal E_2, and 16 received conjugated equine estrogens. Of the 132 women with uteri in situ, 34 received no HRT, 56 received tibolone, 24 received transdermal E_2 with sequential norethisterone acetate, and 18 received conjugated equine estrogens with sequential levonorgestrel.

Intervention(s): Serum VEGF level measurement.

Main Outcome Measure(s): Serum VEGF concentrations.

Result(s): Women who received HRT had higher VEGF concentrations than those not receiving HRT. Among women who received no HRT, those with uterus in situ had higher VEGF levels than did those who had had hysterectomies. Among women who had had hysterectomies, VEGF concentrations were higher in those who received conjugated equine estrogens than in those who did not receive HRT and those who received tibolone or transdermal E_2. Among women with uterus in situ, no difference was found between subgroups.

Conclusion(s): Postmenopausal women with uterus in situ and those who received HRT had higher VEGF concentrations than did those who had had hysterectomies and who did not receive HRT. Among women receiving HRT, those who received conjugated equine estrogens alone had higher VEGF concentrations. This estrogen-mediated increase in serum VEGF concentrations may be a mechanism by which HRT benefits the cardiovascular system. (Fertil Steril® 2000;73:56–60. ©1999 by American Society for Reproductive Medicine.)

Key Words: Vascular endothelial growth factor (VEGF), hormone replacement therapy (HRT), postmenopausal women

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Cyclical fluctuations of vascular endothelial growth factor (VEGF) in the normal menstrual cycle have previously been demonstrated in histologic sections of the ovary (1–4) and in serum (5), but there are no reports on serum VEGF concentrations in postmenopausal women receiving or not receiving hormone replacement therapy (HRT).

There is evidence that a major source of circulating VEGF in women is the reproductive tract (1–6). Vascular endothelial growth factor messenger RNA is present in theca- and granulosa-lutein cells. Serum VEGF levels increase in the luteal phase of the menstrual cycle and VEGF is suppressed by down-regulation of the reproductive axis. Lastly, hypersecretion of VEGF may play a role in the pathogenesis of ovarian hyperstimulation syndrome (6, 7). Because cyclical fluctuations of serum VEGF concentrations are seen during the normal ovulatory cycle (5), we hypothesized that serum VEGF concentrations would decrease after menopause as of the loss of ovarian function. Further, as the uterus is one source of VEGF (3), hysterectomy would also result in lower serum VEGF concentrations.

In the human ovulatory cycle, we also previously demonstrated cyclical fluctuations in

uterine and ovarian Doppler blood flow velocities, which correlated with changes in serum VEGF concentrations. Increasing blood flow velocities and decreasing arterial resistance (pulsatility and resistance indices) were observed with rising VEGF concentrations (5). Doppler blood flow studies in postmenopausal women have demonstrated increased carotid, uterine, and other peripheral artery pulsatility indices, reflecting increased peripheral vascular resistance after menopause (8–10). This putative adverse effect was reversed by treatment with estrogens (8–10). Because VEGF production in the uterus is stimulated by estrogens (11, 12), we hypothesized that increased blood flow velocities in postmenopausal women receiving HRT may be the consequence of an increase in serum VEGF concentrations.

This cross-sectional study was designed to assess VEGF concentrations in healthy postmenopausal women in relation to menopausal HRT. We also aimed to assess whether the presence or absence of a uterus made any difference to serum VEGF concentrations.

MATERIALS AND METHODS

One hundred ninety-nine consecutive postmenopausal women who had been postmenopausal for >5 years were recruited for this cross-sectional study. Ethics committee approval was sought at The Middlesex Hospital and at The Royal Free Hospital. Women with histories of endometriosis, ovarian cysts, uterine fibroids, neoplastic disease, heart disease, or deep venous thromoboembolism were excluded from the study. Forty women had received no menopausal therapy and 159 women had received various forms of menopausal therapy for >5 years, without interruption. Women who had received HRT with interruptions were excluded. One hundred thirty-two patients had uterus in situ and 67 women had had a hysterectomy. All women were assessed clinically, and details of their gynecologic histories, smoking habits, alcohol consumption, and the presence or absence of hypertension were noted.

For analyses, women were divided into four groups: one group consisted of 40 women who had not received any menopausal therapy (6 had had a hysterectomy); the 76 women in the second group received tibolone (2.5 mg/d, Tibolone; Organon Laboratories, Cambridgeshire, United Kingdom) (20 had had hysterectomies); the third group consisted of 49 women who received transdermal E_2 (25 women who had had a hysterectomy received transdermal E_2 [50 μg/d, Estraderm TTS 50; Ciba, Novartis Pharmaceuticals, Camberley, Surrey, United Kingdom] only and 24 women who had uterus in situ also received sequential norethisterone acetate (Estracombi or Estrapak; Ciba, Novartis Pharmaceuticals); and the 34 women in the fourth group received conjugated equine estrogens (16 women who had had a hysterectomy received conjugated equine estrogens [0.625 mg, Premarin; Wyeth Pharmaceutical Laboratories, Maidenhead, Berkshire, United Kingdom] only and 18 women with uteri in situ received conjugated equine estrogens [0.625 mg, Prempak-C; Wyeth Pharmaceutical Laboratories] with sequential norgestrel, 150 μg/d, for 14 days per cycle.

Vascular Endothelial Growth Factor Assay

Fasting blood samples were obtained for serum VEGF measurements, and serum was stored at –70°C until analyzed. Serum VEGF concentrations were measured using enzyme immunoassays (Cytokit Red enzyme immunoassay kits; Peninsula Labs, Inc., College Park, MD), as previously described (5, 7).

Statistical Analysis

Statistical analyses were performed using SPSS for Windows (SPSS, Inc., Chicago, IL). Data are represented as means ± SE. Comparisons between groups were performed by analysis of variance. Post hoc analysis was undertaken using Scheffé's test. *P* values of <.05 were considered significant.

RESULTS

There were no statistically significant differences between women who had had a hysterectomy (n = 67) and those who had not (n = 132) in terms of age (63.3 ± 1.1 vs 64.0 ± 0.7 years), body mass index (24.7 ± 0.5 vs 25.4 ± 0.4), duration of menopausal therapy (7.8 ± 0.6 vs 6.8 ± 0.2 years), prevalence of smoking, alcohol consumption, or hypertension. Similarly, there were no statistically significant differences in these parameters between the various treatment groups (Tables 1 and 2).

Healthy postmenopausal women who did not receive menopausal therapy and who had uterus in situ (n = 34) had significantly higher mean VEGF concentrations (4.15 ± 0.46 ng/mL) than did those who did not receive menopausal therapy and who had had a hysterectomy (n = 6) (2.62 ± 0.87 ng/mL) (*P*<.05) (Fig. 1). The mean serum VEGF concentrations in women receiving menopausal therapy (n = 159) were significantly higher (4.45 ± 0.21 ng/mL) than those in women not receiving HRT (n = 40) (3.09 ± 0.31 ng/mL) (*P*=.006), irrespective of whether the women (in either group) had had a hysterectomy previously.

When all women were considered, differences in mean serum VEGF concentrations between women taking different HRT preparations were not statistically significant. However, women who had uterus in situ, and therefore were receiving combined estrogens with progestogens, had lower VEGF concentrations than did those who had had hysterectomies and were receiving estrogens alone.

Among women who had had a hysterectomy, mean serum VEGF concentrations were significantly higher in those receiving conjugated equine estrogens alone (n = 16) (5.88 ± 0.56 ng/mL) than in women receiving no menopausal therapy (n = 6) (2.62 ± 0.87 ng/mL) or receiving tibolone (n =

TABLE 1

Characteristics of women who had hysterectomies, by menopausal therapy.

Variable	No HRT (n = 6)	Tibolone therapy (n = 20)	TDRE therapy (n = 25)	Treatment with CEE (n = 16)
Age (y)	63.0 ± 3.9	65.6 ± 2.2	62.1 ± 1.8	61.9 ± 2.2
Body mass index	24.8 ± 1.5	24.3 ± 0.8	25.1 ± 0.9	24.7 ± 0.8
Duration of HRT (y)	—	6.2 ± 0.2	8.12 ± 0.6	9.2 ± 1.8

Note: Values are means ± SE. All *P* values were not statistically significant. CEE = conjugated equine estrogens; HRT = hormone replacement therapy; TRDE = transdermal E_2.

Agrawal. Serum VEGF concentrations. Fertil Steril 2000.

20) (4.21 ± 0.55 ng/mL) or transdermal E_2 (n = 25) (4.27 ± 0.42 ng/mL) (P=.02) (Fig. 2).

In contrast, subgroup analysis of postmenopausal women with uteri in situ demonstrated no statistically significant difference in serum VEGF concentrations among treatment groups (Fig. 3).

DISCUSSION

The results of this study suggest that the uterus may contribute to increasing serum VEGF concentrations in postmenopausal women, because mean serum VEGF concentrations were significantly higher in untreated women with uterus in situ than in untreated women who had had a hysterectomy. In addition, women in the study who were treated with menopausal therapy (tibolone, transdermal E_2, or conjugated equine estrogens) had significantly higher mean serum VEGF concentrations than women who were not receiving any menopausal therapy, irrespective of whether the women (in either group) had had a hysterectomy.

The most striking finding, however, was the significantly higher mean serum VEGF concentrations in women who had had a hysterectomy and who received conjugated equine estrogens only, compared with women who had had a hysterectomy and who received other forms of therapy. This conjugated equine estrogen-mediated increase in VEGF concentrations was attenuated when women received conjugated equine estrogens with sequential progestogen. The attenuating effect of progestogen was also observed in women receiving transdermal E_2 with sequential norethisterone acetate, to a similar extent. Tibolone, on the other hand, which is a tissue-selective synthetic steroid with mild estrogenic, progestogenic, and androgenic properties, seems to have only a modest stimulatory effect on serum VEGF levels. Therefore, of the agents we studied, conjugated equine estrogens seem to be the most potent stimulator of VEGF production.

Although it is tempting to conclude that conjugated equine estrogens have a particular effect on VEGF production, it must be accepted that the treatment arms were not randomized and dose equivalents were not sought.

Progestogens are prescribed to postmenopausal women to protect the endometrium from hyperplastic and potentially carcinomatous effects of prolonged and unopposed estrogen replacement therapy. Progestogens act by down-regulating estrogen receptors. Thus, estrogen-induced changes in hepatic metabolism of cholesterol-transport proteins are reduced when progestogens are added to estrogen replacement therapy. Progesterone receptors are present in the arterial wall, and there is evidence that progestogen cellular actions in arteries are mediated through P receptors as well as through down-regulation of the E_2 receptor. Progestogen has been shown to affect arterial function and can stabilize arteries in a state of vasomotor instability. Progestogens also induce vasoconstriction of estrogenized vessels (13, 14). Therefore, the main concerns about progestogens' compro-

TABLE 2

Characteristics of women with uteri in situ, by menopausal therapy.

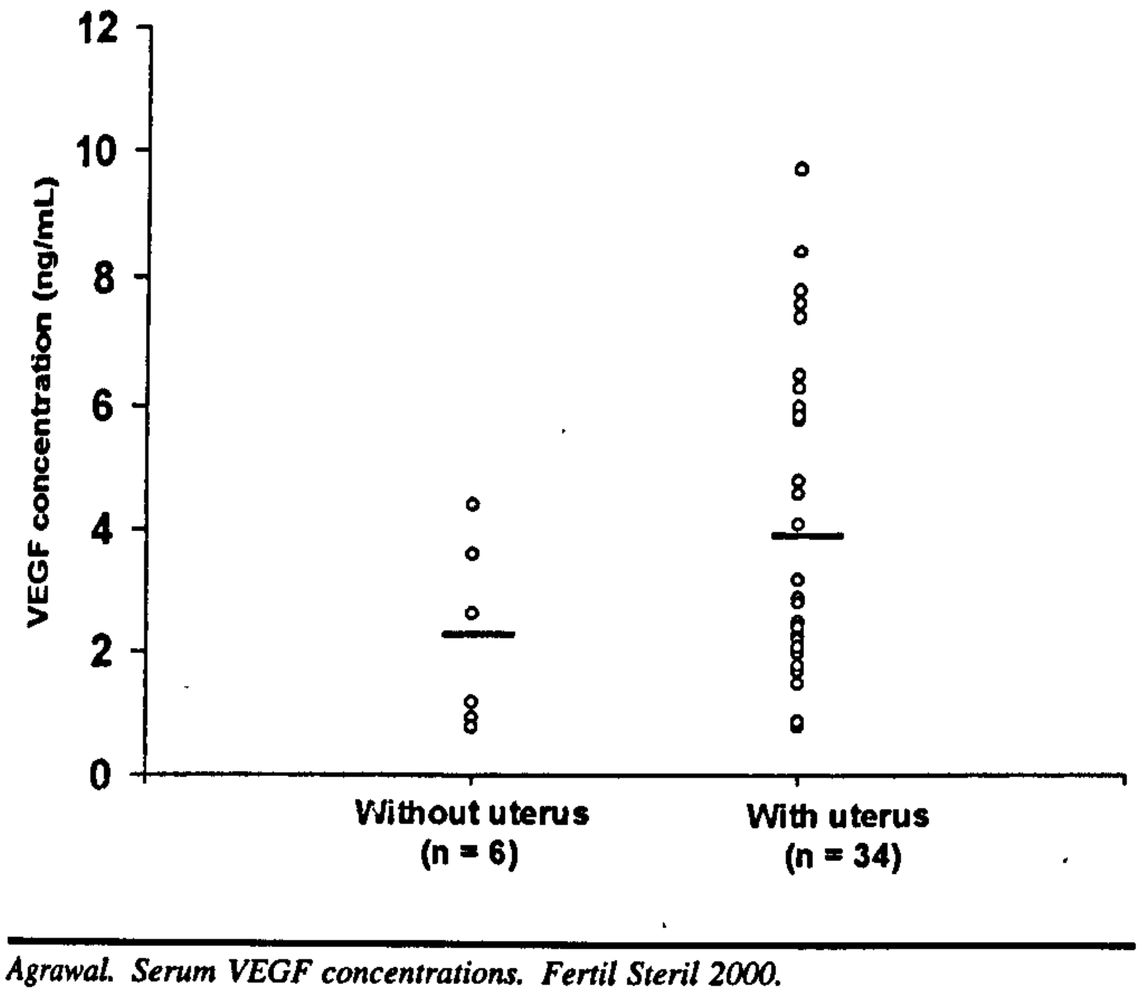
Variable	No HRT (n = 34)	Tibolone therapy (n = 56)	TDRE therapy (n = 24)	Treatment with CEE (n = 18)
Age (y)	65.3 ± 1.3	66.2 ± 0.9	64.3 ± 1.3	64.2 ± 1.7
Body mass index	26.0 ± 0.8	25.3 ± 0.5	25.7 ± 0.8	23.7 ± 1.8
Duration of HRT (y)	—	6.6 ± 0.2	7.3 ± 0.4	7.8 ± 0.9

Note: Values are means ± SE. All *P* values were not statistically significant. CEE = conjugated equine estrogens; HRT = hormone replacement therapy; TRDE = transdermal E_2.

Agrawal. Serum VEGF concentrations. Fertil Steril 2000.

FIGURE 1

Serum vascular endothelial growth factor (VEGF) concentrations in healthy postmenopausal women not receiving hormone replacement therapy who had or did not have a hysterectomy. *Horizontal bars* represent means. $P<.05$ (without uterus vs with uterus).



Agrawal. Serum VEGF concentrations. Fertil Steril 2000.

mising the cardioprotective actions of estrogens rest in the recognition that effects of estrogen on arterial physiology are important in preventing ischemic events and that progestogens could have an adverse effect on these actions.

Our findings of the attenuating effect on serum VEGF concentrations of progestogens added to conjugated equine estrogens are consistent with a recent demonstration that interrupted progestogen HRT reduced VEGF activity and subsequent angiogenesis within the human endometrium by allowing frequent progestogen withdrawal to occur (15). Rogers and colleagues (16) also demonstrated that microvascular activity in the endometrium is increased by long-term, continuous exposure to progestogen but not by interrupted progestogen therapy.

These data are supported by findings of studies of the effects of HRT on coronary arteries in primates. Williams and colleagues (17) demonstrated that conjugated equine estrogens restored vasodilatory response to arteries stimulated with acetycholine because of stimulation of vascular endothelium, which results in release of nitric oxide, but this response was inhibited by cyclical medroxyprogesterone acetate therapy and entirely eliminated when animals received continuous medroxyprogesterone acetate therapy. Similar findings were reported by Miyagawa and colleagues (18). Gorodeski et al. (19) reported that in the rabbit, P counteracted the beneficial effects of estrogen on coronary blood flow.

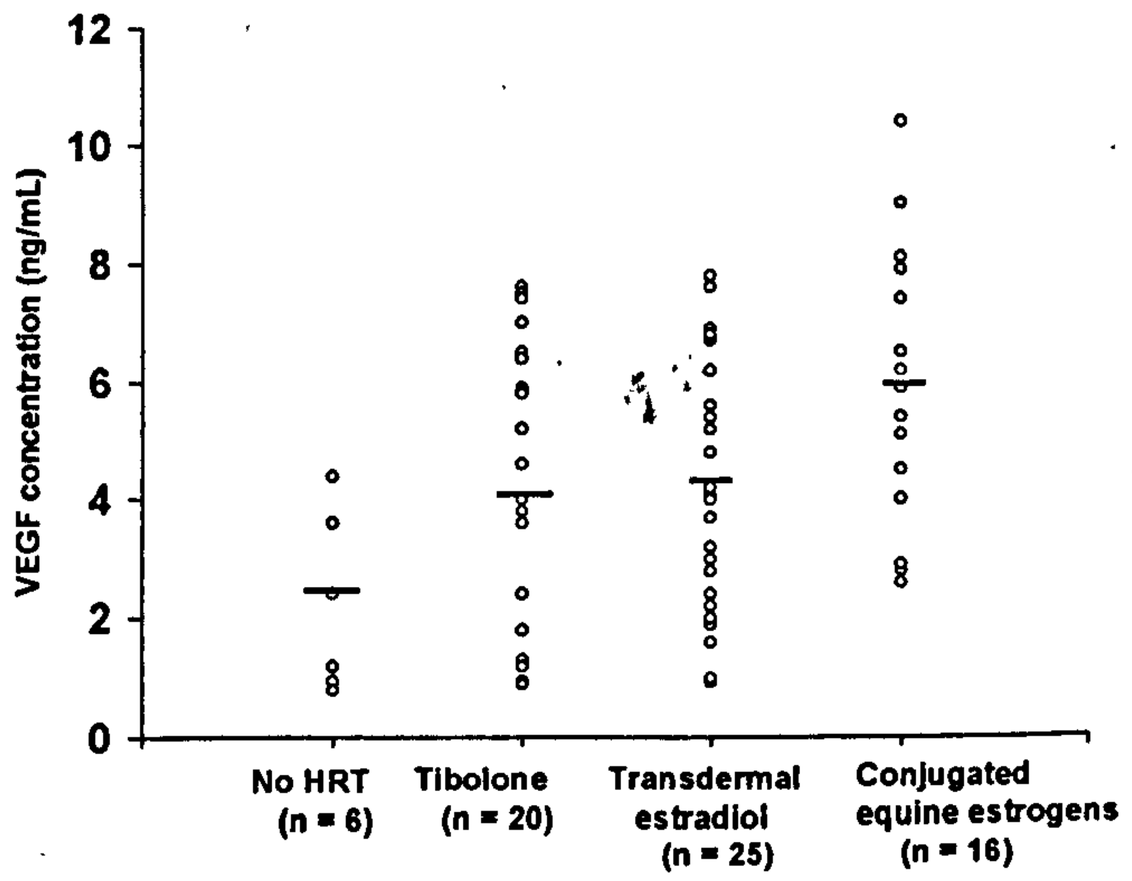
Although numerous studies support the role of estrogens in cardiovascular protection (20–22), the magnitude of this effect and the mechanisms by which estrogens achieve their beneficial effects on cardiovascular function are not fully understood. The beneficial effects of estrogens on lipid or lipoprotein profiles account for, at most, 20%–30% of the reported cardiovascular benefits of estrogen replacement therapy (23). Recent studies have focused on other aspects of estrogenic action that may explain the cardiovascular benefits, such as direct effects on the vessel wall, glucose metabolism, and changes in fibrinolytic activity (24).

Coronary angiogenesis is an important homeostatic response to myocardial ischemia. The number of VEGF receptors is increased by ischemia, thus allowing the effect of increased serum VEGF concentrations to be expressed. Vascular endothelial growth factor has potent angiogenic activity, and its primary role is to promote endothelial growth. It has been shown to induce endothelium-dependent relaxation in both canine coronary arteries and rabbit renal arteries, both in vivo and in vitro, most likely by stimulating nitric oxide and prostacyclin release (25–27). Intracoronary administration of recombinant VEGF in dogs also enhanced development of small coronary arteries supplying ischemic myocardium, resulting in marked augmentation of maximal collateral blood flow delivery (28).

Angiogenic effects of exogenously administered VEGF on collateral hind limb arterial and capillary blood flow and coronary artery blood flow after ischemic injury have also been demonstrated (29, 30).

FIGURE 2

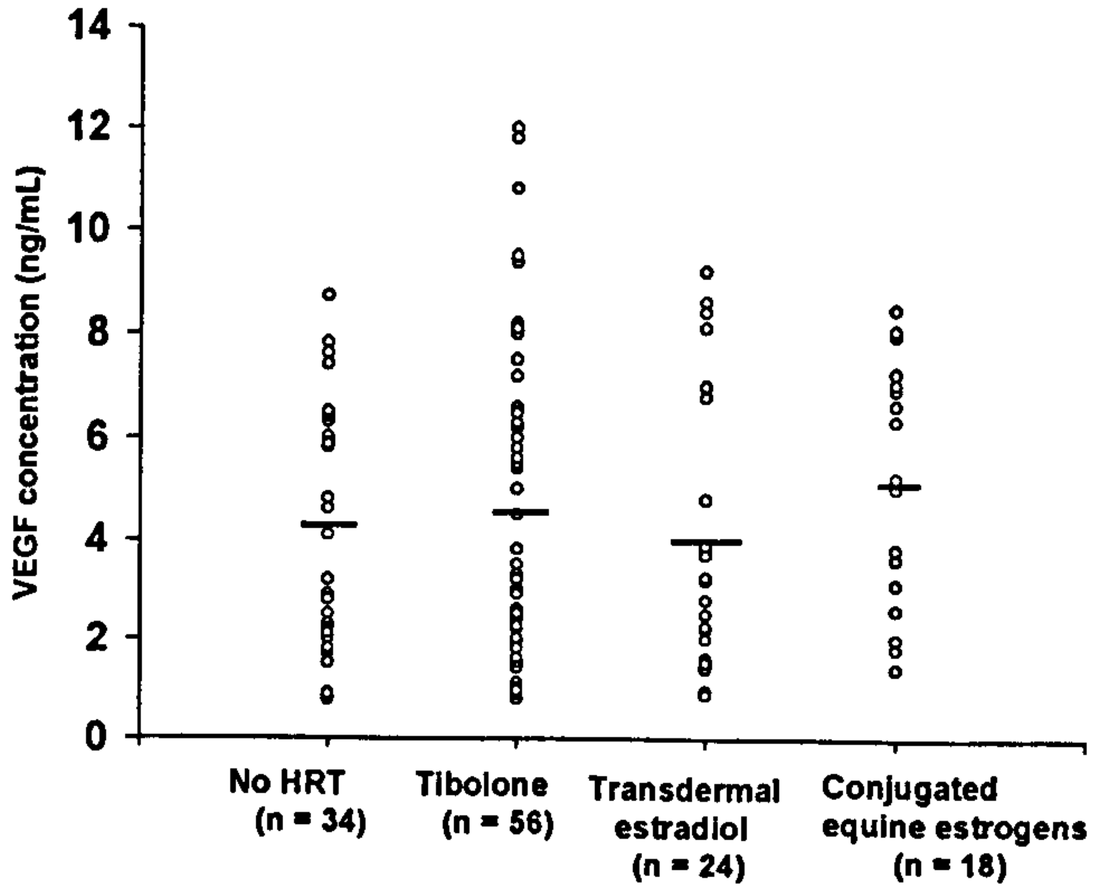
Serum vascular endothelial growth factor (VEGF) concentrations in postmenopausal women who had a hysterectomy. *Horizontal bars* represent means. $P=.02$ (no hormone replacement therapy [HRT], tibolone therapy, and transdermal E_2 therapy vs treatment with conjugated equine estrogens).



Agrawal. Serum VEGF concentrations. Fertil Steril 2000.

FIGURE 3

Serum vascular endothelial growth factor (VEGF) concentrations in postmenopausal women with uteri in situ. *Horizontal bars* represent means. *P* = not significant. HRT, hormone replacement therapy.



Agrawal. Serum VEGF concentrations. Fertil Steril 2000.

It is therefore possible that the observed estrogen-associated increase in serum VEGF concentrations, documented for the first time in the present study, could be one of the mechanisms by which estrogens exert a beneficial effect on the cardiovascular system.

In conclusion, mean serum VEGF concentrations are higher in postmenopausal women with uterus in situ than in women who have had a hysterectomy and have not received any HRT. In addition, mean serum VEGF concentrations are higher in postmenopausal women receiving HRT, particularly those taking conjugated equine estrogens alone. It is possible that this increase in VEGF concentrations contributes to the beneficial effects of menopausal therapy on the cardiovascular system.

It seems, however, that the addition of sequential progestogen attenuates the increase in serum VEGF concentrations observed with treatment with conjugated equine estrogens alone.

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